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(54) Title: MONOCLONAL PARATOPIC MOLECULE DIRECTED TO HUMAN GANGLIOSIDE GD2

(57) Abstract

A hybridoma having ATCC accession number HB9118 that secretes a monoclonal paratopic molecule that immunoreacts with ganglioside GD2, compositions containing that paratopic molecule, as well as methods and a system of using the paratopic molecule and its compositions.

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# MONOCLONAL PARATOPIC MOLECULE DIRECTED TO HUMAN GANGLIOSIDE GD2

## Description

### Technical Field

The present invention relates to monoclonal paratopic molecules, and more particularly to a monoclonal paratopic molecule that reacts with human tumor cells expressing ganglioside GD2.

### Background of the Invention

Neuroectodermal tumors are highly malignant, and include neuroblastomas, small cell cancinoma of the lung, gliomas, neuroblastomas and melanomas. Of the neuroectodermal tumors, neuroblastomas occur during infancy and early childhood. Except for Wilms' tumor, they are the most common retroperitoneal tumors in children. Neuroblastomas arise most commonly in the adrenal medulla, but they may also develop in other sympathetic ganglia within the thorax or abdomen. These tumors metastasize early with widespread involvement of lymph nodes, liver, bone, lung and marrow. The prognosis is often good when the tumor is diagnosed prior to obvious metastasis, but with metastasis, prognosis is poor despite the extensive use of radical surgery, deep X-ray therapy, and chemotherapeutic agents.

Several antigenic determinants have recently been detected on neuroblastoma cells with monoclonal antibodies (Mabs). See Seeger, Ann. Intern. Med., 97, 873 (1982); Wikstrand et al., Cancer Res., 42, 267 (1982); Wikstrand et al., J. Neuroimmunlogy, 3, 43 (1982); Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76, 4913 (1979); Liao et al., Eur. J. Immunol., 11, 450 (1981); Seeger et al., Cancer Res., 4, 2714 (1981); Kennett et al., Advances in Neuroblastoma Research, p. 209, Raven Press, New York (Evans ed.)

(1980); Seeger et al., J. Immunol., 128, 983 (1982); Kemshead et al., Pediatr. Res., 15, 1282 (1981).

A panel of such antibodies has been reported to be helpful in the differential diagnosis of neuroblastoma and lymphoblastic disorders, Kemshead et al., Pediatr. Res., 15, 1282 (1981); Kemshead et al., Lancet, i, 12 (1983). In these same studies, antibodies were used either in immunoperoxidase assays with tumor tissue sections or in direct immunofluorescence assays to detect tumor cells in bone marrow aspirates. However, there have not been any reports describing the use of antibodies to neuroblastoma-associated antigens to detect elevated amounts of these antigens in the sera of patients and normal children.

The effective use of Mabs directed to any tumor-associated antigens as diagnostic reagents depends on the quantity, expression and chemical nature of the corresponding antigen. In this regard, Mabs directed to tumor-associated gangliosides have been useful in defining antigens associated with melanoma, neuroblastoma, colon carcinoma, and adenocarcinoma, Hakomori et al., J. Natl. Cancer Inst., 71, 231 (1983). One of these antibodies was reported to detect a ganglioside antigen shed into the serum of patients with colon carcinomas, Koprowski et al., Science, 212, 53 (1981).

Some of the above neuroblastoma-associated antigens are present in fetal neural tissues whereas others are expressed by both fetal and adult neural tissues. Seeger, Ann. Intern. Med., 97, 873 (1982).

Most of the monoclonal antibodies utilized to detect the neuroblastoma-associated antigens are not restricted in their reactivity to neuroectodermal tumors like melanoma and glioma but also recognize

common antigens on other malignancies such as a variety of sarcomas and leukemias, Seeger, Ann. Intern. Med., 97, 873 (1982). In addition, only some of the antigenic structures on neuroblastoma cells recognized by monoclonal antibodies have been partially characterized by immunochemical means. Thus, a monoclonal antibody designated Mab 390 was reported to react with an antigenic determinant of human Thy-1 that had a molecular weight of 25,000 daltons. Seeger et al., J. Immunol., 128, 983 (1982).

Another Mab, designated $A_2B_5$, was reported to recognize a GQ ganglioside on neurons, Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76, 4913 (1979). A human monoclonal antibody produced in vitro by a lymphoblast cell line from a melanoma patient was also reported to react with a GD2 ganglioside present on neuroectoderm-derived tumors, Cahan et al., Proc. Natl. Acad. Sci. USA, 79, 7629 (1982).

U.K. patent application GB 2121417A describes a monoclonal antibody designated Cal that immunoreacts with several malignant tumor cells. The antigen with which that antibody reacts is reported to be on glycoproteins having molecular weights of about 390,000 and 350,000 in SDS acrylamide gels, and the antigen reportedly includes both polysaccharide and protein components.

From a biological point of view, gangliosides are of considerable interest since they have been implicated in a variety of cellular functions, including cell-cell adhesion and communication, as well as cell-substrate interactions, Hakomori et al., J. Natl. Cancer Inst., 71, 231 (1983). Recent studies have emphasized the importance of gangliosides for tumor growth

regulation by demonstrating differences in ganglioside composition among cells expressing various degrees of tumorgenicity, Itaya et al., Proc. Natl. Acad. Sci. USA, 73, 1568 (1976). Consequently, the use of monoclonal antibodies directed to ganglioside determinants can aid in further delineating the role of gangliosides in these processes.

Most of the monoclonal antibodies directed against neuroblastoma-associated antigens that have been reported thus far, Wikstrand et al., Cancer Res., 42, 267 (1982); Wikstrand et al., J. Neuroimmunology, 3, 43 (1982); Eisenbarth et al., Proc Natl. Acad. Sci. USA, 76, 4913 (1979), recognize a common antigenic determinant on fetal tissues, especially fetal brain, as well as on adult brain and other neural tissues. In addition, cross-reactions of such antibodies have also been reported with normal kidney, fibroblasts, myoblasts, and thymocytes, Seeger et al., Cancer Res., 4, 2714 (1981), and Seeger et al., J. Immunol., 128, 983 (1982), with islet cells, Eisenbarth et al., Proc. Natl. Acad. Sci. USA, 76, 4913 (1979) and with spleen cells, Wikstrand et al., Cancer Res., 42, 267 (1982). Furthermore, some of the monoclonal antibodies reported in the literature are not only restricted in their reactivity to neuroectodermal tumors, such as neuroblastoma, melanoma and glioma, but also show binding to some forms of leukemia, osteogenic sarcoma, rhabdomyosarcoma, leiomyosarcoma and even to carcinomas of the lung and breast, Seeger, Ann. Intern. Med., 97, 873 (1982).

A series of monoclonal antibodies that immunoreact with a number of tumor cell-associated antigens is reported in European patent application

0 104 014. Of those antibodies, those designated Ab A010, Ab AJ9 and Ab AJ2 were reported to have particularly high titers against neuroblastoma cell lines SK NMC and SK NSH. Of those antibodies, Ab A010 was also reported to react with a determinant present in adult and fetal brain, but not in adult and fetal skin fibroblasts, kidney epithelial cells or melanocytes. The determinant bound by Ab A010 was further reported to be heat labile. Antibody Ab AJ9 was said to recognize heat-labile determinants on most normal and malignant cells. Finally, Ab AJ2 was reported to react with a determinant on all nucleated human cells examined, both normal and malignant.

In addition, Cheresh et al., J. Cell. Biol., 102, 688 (1986) have reported an IgM monoclonal antibody designated Mab 126 that immunoreacts with ganglioside GD2. That antibody, while reacting with ostensively the same determinant as does a paratopic molecule of the present invention, exhibits a somewhat different reactivity pattern from that of a paratopic molecule of the present invention, as is illustrated hereinafter.

Heterogeneity of neuroblastomas with regard to cell surface antigenic expression has been reported in Seeger, Ann. Intern. Med., 97, 873 (1982); Kemshead et al., Pediatr. Res., 15, 1282 (1981); Kemshead et al., Int. J. Cancer, 27, 447 (1981); and, Kemshead et al., Proc. Am. Assoc. Cancer Res., 2, 399 (1981). As discussed in these publications, Mab $A_2B_5$ failed to react with some human neuroblastoma lines tested, and quantitative differences in antigenic expression were observed between different cell cultures. Analysis of tumor cells in heavily infiltrated bone marrow aspirates indicated that only 70 percent of the samples reacted

with $A_2B_5$, suggesting that the heterogeneity seen in the expression of antigen on cell lines is paralleled in fresh tumor material, Kemshead et al., Int. J. Cancer, 27, 447 (1981).

Summary of the Invention

The present invention contemplates a monoclonal paratopic molecule such as an intact antibody, methods of preparing and using the paratopic molecule, and diagnostics utilizing the paratopic molecule. The monoclonal paratopic molecule is produced by a hybridoma formed by the fusion of a myeloma cell line and a lymphocyte that produces antibodies that react with disialoganglioside GD2, usually referred to herein as "ganglioside GD2" or "GD2". The monoclonal paratopic molecule reacts with ganglioside GD2 and with cells expressing ganglioside GD2 thereon.

In one aspect of the invention, an IgG3 murine monoclonal antibody and its antibody combining site-containing polypeptide portions (a monoclonal paratopic molecule) are contemplated that immunoreact with (bind to) ganglioside GD2. The intact monoclonal antibody (designated Mab 14.18) is secreted (produced) by hybridoma ATCC HB 9118. The paratope portion of monoclonal antibody Mab 14.18 reacts with cells that express ganglioside GD2 and does not react with cells that do not express ganglioside GD2. For example, Mab 14.18 reacts with neuroblastoma cell lines, melanoma cell lines, small cell lung carcinoma cell lines, glioblastoma cell lines U87MG, U138MG and U373MG and lung adenocarcinoma cell line T291, all of which express ganglioside GD2. Mab 14.18 is substantially free from reaction with squamous carcinoma cell lines USCLS-1, SK-MES-1 and CALU-6, pancreatic carcinoma

cell lines COLO 357, SG and FG, leukemic cell line Molt-4, monocytic lymphoma cell line U937, and adenocarcinoma cell line UCLA-P3, that do not express the ganglioside GD2.

Monoclonal antibody 14.18 and its paratope-containing polypeptide portions; i.e., the monoclonal paratopic molecule of this invention, are useful for assays to detect, diagnose and monitor tumors and other cells having ganglioside GD2 expressed thereon, as well as to assay compositions containing that ganglioside.

In particular, one embodiment of this invention relates to a diagnostic system, typically in kit form, for assaying for the presence ganglioside GD2. The system includes in at least one container, as an active ingredient, an effective amount of a monoclonal paratopic molecule of this invention such as Mab 14.18. The system can also contain an indicating means, and other reagents.

Another aspect contemplates a method of analysis for the presence of ganglioside GD2. In accordance with that method, an amount of a paratopic molecule of this invention effective to immunoreact with ganglioside GD2 sought is admixed and contacted with a sample to be assayed for the presence of that ganglioside. The admixture is maintained under biological assay conditions for a predetermined period of time sufficient for the paratopic molecule to immunoreact with any ganglioside GD2 present in the sample to form an immunoreactant or complex. The presence of the immunoreactant is then determined, as can be the amount of immunoreactant. In preferred practice, this assay is carried out as a solid phase assay in which the sample to be assayed is affixed to a solid phase matrix such as a slide, a microtiter

plate well or the internal cells of a tumor, and the paratopic molecule is in an aqueous composition.

The presence of the immunoreactant can be determined by an indicating means that is linked to a second molecule that binds to the paratopic molecule, or the indicating means can be linked directly to the paratopic molecule.

Thus, one embodiment of the above diagnostic method is in vivo imaging of cells such as tumor cells having ganglioside GD2 expressed thereon, while another embodiment is a more usually used immunodiagnostic method such as an ELISA or RIA.

In another aspect of the present invention, a hybridoma for the production (secretion) of the above described monoclonal antibody Mab 14.18, that reacts with human cells having ganglioside GD2 expressed thereon, is contemplated. The hybridoma is designated ATCC HB 9118.

A further aspect of the present invention contemplates a method of preparing the above described monoclonal antibody. The method comprises growing the hybridoma ATCC HB 9118 in a suitable culture medium and recovering the antibody from the medium containing said hybridoma. That suitable medium can be an in vitro cell culture medium or the body of a laboratory animal from which the antibody can be recovered from the malignant ascites or serum.

As it is highly cytotoxic to tumor cells having ganglioside GD2 expressed thereon in both complement-dependent cytotoxicty assays and antibody-dependent cellular cytotoxicity assays, the monoclonal paratopic molecule of the present invention can be used for killing such tumor cells. In this regard, a composition for killing tumor cells having ganglioside GD2 expressed thereon and a method for doing same are contemplated.

The composition comprises, as an active ingredient, the above-described monoclonal paratopic molecule as the intact antibody Mab 14.18 dispersed in an aqueous physiologically tolerable diluent. The monoclonal paratopic molecule is present in an amount sufficient to kill tumor cells expressing ganglioside GD2 on their surfaces when the antibody and tumor cells are admixed with either complement or effector cells. The composition can further include (a) an additional cytotoxic agent such as a drug linked to the antibody, (b) complement from an appropriate animal species, or (c) effector cells such as peripheral blood mononuclear cells (PBM) from an appropriate animal species.

A method of killing tumor cells that express ganglioside GD2 on their cell surfaces comprises contacting the tumor cells with a cytotoxic amount of the above composition in the presence of (i) complement, or (ii) of effector cells such as PBM, or (iii) both complement and effector cells, and maintaining that contact for a predetermined period of time sufficient to kill tumor cells. In an alternative method, the monoclonal paratopic molecule of the composition is utilized as a carrier for a linked cytotoxic agent, and that composition is contacted with the tumor cells and maintained as described.

The present invention provides several benefits and advantages.

One benefit of the present invention is that the monoclonal paratopic molecule of the present invention is useful, inter alia, for effective immunotherapy of malignancies that express ganglioside GD2 on the surfaces of tumor cells.

Another benefit of the invention is that the monoclonal paratopic molecule provides a diagnostic means for identifying cells that express ganglioside GD2.

One advantage of the present invention is that the monoclonal antibody of the invention can mediate tumor cell killing by use in combination with either complement or effector cells.

Other benefits and advantages of the present invention will become readily apparent to those skilled in the art from the following detailed description that follows.

Brief Description of the Drawings

In drawings forming a portion of the disclosure of this invention:

FIGURE 1 is a copy of a chromatogram that illustrates the staining by resorcinol of total gangliosides separated on thin layer chromatography (TLC). Total gangliosides extracted from a 5 microliter (ul) packed cell equivalent of the cultured human small cell lung carcinoma (SCCL) cell lines NCI H-69 or NCI H-82 were separated on thin layer chromatography (TLC). Gangliosides from those two cell lines were stained as described hereinafter in Section III, and are shown in lanes A and B, respectively. The migration of different ganglioside standards on the TLC separation is indicated by brackets to the left of the chromatogram.

FIGURE 2 is a copy of a chromatogram that illustrates the immunostaining of gangliosides separated on TLC using monoclonal antibodies. Total gangliosides extracted from a one ul packed cell equivalent of the cultured tumor cell lines NCI H-69, NCI H-82 or NCI H-460 were separated on TLC and are each shown in the set of lanes A,D & G or B,E & H or

C,F & I, respectively. Lanes A-C show immunostaining with the anti-GD2 Mab 126, lanes D-F show immunostaining with the anti-GD2 Mab 14.18 and lanes G-I show immunostaining with an anti-GM2 Mab 5-3 performed as described hereinafter in Section III. The migration of different ganglioside standards on TLC separation is indicated to the left of the chromatogram by brackets.

FIGURE 3 is a copy of a photomicrograph showing the detection of ganglioside GD2 on frozen human SCCL biopsy tissue by the immunoperoxidase technique described hereinafter in Section III. Panel A shows immunoperoxidase staining using the monoclonal antibody Mab 14.18 and Panel B shows staining using hematoxylin plus eosin.

FIGURE 4 is a graph that illustrates the complement-dependent in vitro cytotoxicity (CDC) mediated by Mab 14.18 against SCCL cells. The specific lysis of T293 human SCCL cells was measured by the percent release over time of incorporated $^{51}Cr$ label in the presence of human complement and Mab 14.18 as described hereinafter in Section III. Significant lysis was not observed in parallel cultures containing human complement and a control, non-binding antibody.

FIGURE 5 is a bar graph illustrating the antibody-dependent cellular cytotoxicity (ADCC) mediated by Mab 14.18 and normal human peripheral blood mononuclear (effector) cells against SCCL cells. The specific lysis of T293 SCCL (target) cells was measured by the release of incorporated $^{51}Cr$ label when challenged with effector cells from either of two healthy donors in the presence (open bars) or absence (hatched bars) of Mab 14.18 as described hereinafter in Section III. The effector to target cell ratio varied from 50 to 200.

## Detailed Description of the Invention

### DEFINITIONS

The term "antibody" refers to a molecule that is a member of a family of glycosylated proteins called immunoglobulins that can specifically combine with an antigen. Such an antibody combines with its antigen by a specific immunologic binding interaction between the antigenic determinant of the antigen and the antibody combining site of the antibody.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen. Using the nomenclature of Jerne, Ann. Immunol. (Inst. Pasteur), 125C, 373 (1974) an antibody combining site is usually referred to herein as a "paratope".

Antibody combining site-containing (paratope-containing) polypeptide portions of antibodies are those portions of antibody molecules that contain the paratope and bind to an antigen, and include the Fab, Fab', $F(ab')_2$ and F(v) portions of the antibodies. Fab and $F(ab')_2$ portions of antibodies are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous and Dixon. Fab' antibody portions are also well known and are produced from $F(ab')_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. Intact antibodies are preferred, and are utilized as illustrative of the monoclonal paratopic molecule of this invention.

The word "antigen" has been used historically to designate an entity that is bound by an antibody, and also to designate the entity that induces the production of the antibody. More current usage limits the meaning of antigen to that entity bound by an antibody, whereas the word "immunogen" is used for the entity that induces antibody production. Where an entity discussed herein is both immunogenic and antigenic, it will generally be termed an antigen.

The phrase "antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site. The Jerne nomenclature defines an antigenic determinant as an "epitope".

The term "biologically active" refers at least to the ability to specifically bind antigen or specific antibody combining site, although other general or effector capability may be present as well.

Biological activity of a paratopic molecule containing an antibody combining site is evidenced by the immunologic reaction of the antibody paratope (combining site) with its epitope (antigenic determinant) upon their admixture in an aqueous medium to form an immunoreactant, at least at physiological pH values and ionic strengths. Preferably, biological activity occurs under biological assay conditions; i.e., those conditions wherein a paratope-containing molecule of this invention binds to its epitope within a pH value range of about 5 to about 9, at ionic strengths such as that of distilled water to that of about one molar sodium chloride, and at temperatures of about 4 degrees C to about 45 degrees C.

"ELISA" refers to an enzyme-linked immunosorbent assay that employs an antigen or antibody bound to a solid phase and an enzyme-antibody or enzyme-antigen conjugate to detect and quantify the amount of antigen or antibody present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043, which are incorporated herein by reference.

"Enzyme" refers to a protein capable of accelerating or producing by catalytic action some change in a substrate for which it is often specific.

"Immunoreactant" as used herein refers to the product of an immunological reaction; i.e., that entity produced when an antigen is immunologically bound by an antibody or a molecule containing a paratope. An immunoreactant is therefore a specific type of complex formed between molecules.

The terms "indicating means", "indicating group" or "label" are interchangeably used herein to include single atoms, molecules and enzymes that are either directly or indirectly involved in the production of a detectable signal to indicate their presence. Substantially any indicating means that can be linked to or incorporated in a antibody is useful herein, and those indicating means can be used alone or in conjunction with additional reagents. Such indicating groups or labels are themselves well-known in immunochemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel antibodies, methods and/or systems.

The term "intact antibody" is used herein to distinguish a complete molecule secreted by a cell from other, smaller, molecules that also contain the paratope necessary for biological activity in an immunoreaction with an antigen.

The paratopic molecules useful in the present invention are monoclonal paratopic molecules. A "monoclonal antibody" (Mab) is a antibody produced by clones of a hybridoma that secretes but one kind of antibody molecule, and a monoclonal paratopic molecule is a monoclonal antibody or a paratope-containing polypeptide portion thereof, as is discussed below. The hybridoma cell is fused from an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, Nature, 256, 495-497 (1975), which description is incorporated herein by reference.

The terms "monoclonal paratopic molecule" or "paratopic molecule" are used interchangeably and collectively herein to refer to the genus of molecules that contain a combining site of a monoclonal antibody, and include an intact monoclonal antibody, a substantially intact monoclonal antibody and an antibody binding site-containing portion of a monoclonal antibody. The intact monoclonal antibody designated Mab 14.18 is a paratopic molecule of this invention as are portions of that intact antibody that include the paratope. The terms "monoclonal paratopic molecule" or "paratopic molecule" are used herein when a generic biolgically active molecule containing the paratope of Mab 14.18 is intended, while the term "Mab 14.18" or an equivalent term that includes the numerals "14.18" is used where the specific intact antibody produced by hybridoma ATCC HB 9118 is intended.

The words "secrete" and "produce" are often used interchangeably in the art as to cells from which antibody molecules are obtained. Cells that produce antibodies may, however, not secrete those molecules into their environment. The hybridoma cells of interest herein secrete monoclonal antibodies into their environment. Nevertheless, such cells are often referred to herein as "antibody-producing" cells, and their antibodies are sometimes referred to as being "produced" in keeping with the phrase utilized in the art. Paratope-containing polypeptide portions of Mab 14.18 are similarly referred to herein as being "produced" or "secreted", although it is to be understood that such molecules are prepared from antibodies that are themselves "produced" or "secreted".

The terms "supernate" and "supernatant" are used interchangeably herein and refer to the in vitro liquid medium in which cells are cultured. Monoclonal paratopic molecules produced by the hybridoma cultures of interest herein are secreted into their culture medium environment. Therefore the culture medium supernate is one preferred source of the monoclonal paratopic molecule and is readily obtainable free from hybridoma cells by well known techniques. Exemplary of such techniques is low speed centrifugation to sediment cells out of the liquid medium. Monoclonal paratopic molecules can alternatively be obtained from ascites tumor fluid of (ascites fluid) laboratory animals into which the hybridoma tissue was introduced. Both methods are described hereinafter.

## I. GENERAL DISCUSSION

The present invention is directed to a monoclonal paratopic molecule and to methods of

preparing and using same, as well as diagnostics utilizing the paratopic molecule. The monoclonal paratopic molecule reacts with human tumors having ganglioside GD2 antigen expressed thereon. Such tumors are typically of neuroectodermal origin.

A. The Hybridoma

To form the hybridoma from which a monoclonal paratopic molecule of this invention is produced, myeloma cell line Sp2/0 was fused with lymphocytes that produced antibodies that react with ganglioside GD2. In particular, splenocytes from a mouse immunized with neubroblastoma cell line LAN-1 that express ganglioside GD2 on their cell surfaces were utilized.

The hybridoma denominated Mab 14.18 was deposited with the American Type Culture Collection, Rockville, Maryland on June 4, 1986, was given the designation, ATCC HB 9118, and is the hybridoma of the present invention. All hybridomas and their monoclonal antibodies described herein by an "ATCC" designation are on deposit with the American Type Culture Collection.

The present deposit was made in compliance with the Budapest Treaty requirements that the duration of the deposit should be for 30 years from the date of deposit or for 5 years after the last request for the deposit at the depository or for the enforceable life of a U.S. patent that matures from this application, whichever is longer. The hybridoma will be replenished should it become non-viable at the depository.

B. The Monoclonal Paratopic Molecule

A monoclonal paratopic molecule of this invention as exemplified by the murine monoclonal antibody designated Mab 14.18 recognizes a

disialoganglioside antigen that is strongly expressed on all neuroblastoma, melanoma and small cell lung carcinoma (SCCL) cell lines and tissues screened using the ELISA (enzyme-linked immunosorbant assay) technique. It is believed that the ganglioside antigen recognized by Mab 14.18 is expressed on substantially all neuroblastoma, melanoma and SCCL cells. Put differently, it is believed that Mab 14.18 reacts with ganglioside GD2 and therefore it also reacts with substantially all cells that express ganglioside GD2 on their surfaces. Mab 14.18 is particularly reactive with neuroectodermal-derived and SCCL tumor cells that express ganglioside GD2.

In ELISA, the paratopic molecules of this invention failed to react with nearly all other lymphoma, sarcoma, and carcinom cell lines with the exception of those mentioned hereinabove.

The screening of fresh frozen normal and malignant human tissues using the immunoperoxidase technique again indicated that Mab 14.18 had its greatest reactivity with melanoma and SCCL tumor tissues. With normal tissues, there was only a faint positive reactivity with spleen, lung and skin tissues.

Mab 14.18 differs from previously reported monoclonal antibodies directed against neuroblastoma-associated antigens since it is more restricted in its tissue-binding capabilities than are other monoclonal antibodies directed to neuroblastomas. Thus, cross-reactions of such previously reported antibodies with normal kidney, fibroblasts, myoblasts, thymocytes, islet cells and spleen cells are minimal with Mab 14.18. Furthermore, Mab 14.18 is substantially restricted in its reactivity to neuroectodermal tumors, such as

neuroblastoma, melanoma and glioma, and to SCCL, in contrast some of the similarly derived monoclonal antibodies reported in the literature that also exhibit binding to some forms of leukemia, osteogenic sarcoma, rhabdomyosarcoma, leiomyosarcoma and carcinomas of the breast and of the lung other than SCCL.

A monospecific human cell-derived antibody, (anti-OFA I-2), produced in vitro by a lymphoblast cell line originating from a human melanoma patient has been reported to detect a GD2 ganglioside on human melanoma, glioma and neuroblastoma cells. Similar to Mab 14.18, anti-OFA I-2 was reported not to bind to a variety of cell lines derived from carcinomas and from different lymphoid tumors, Cahan et al., Proc. Natl. Acad. Sci. (USA), 79, 7629 (1982); and, Irie et al; Proc. Natl. Acad. Sci. (USA), 79, 5666 (1982).

A monoclonal antibody such as Mab 14.18 has a particular advantage over a human cell-derived monoclonal antibody such as anti-OFA I-2 that recognizes a similar antigenic determinant when used for immunoperoxidase assays of human tissues. The advantage stems from the finding that the anti-human secondary antibody required for such assays causes a large amount of non-specific background reactivity. Use of Mab 14.18 does not provide such a background because it is a murine antibody that does not utilize an anti-human second antibody in such assays. Thus, the human Mab OFA I-2 has not been widely used for such assays.

In view of these characteristics, Mab 14.18 is useful for the diagnosis of small round cell tumors in children; i.e., to distinguish neuroblastoma from lymphoblastic lymphoma, leukemia,

rhabdomyosarcoma, and Ewing sarcoma. In this regard, it is well known that the differential diagnosis of these tumors has heretofore often been difficult, Kemshead et al., Lancet, 118, 12 (1983); Reynolds et al., Cancer, 48, 2088 (1981); and, Raney et al., J. Ped., 89, 433 (1976).

A clinical use of Mab 14.18 is further indicated by previous results that clearly demonstrate the presence of elevated levels of the ganglioside GD2 in the sera of most patients with neuroblastoma compared to sera of normal children or patients with other pediatric tumors, Schultz, et al., Cancer Res., 44 5914 (1984). These results suggest that the level of GD2 antigen in the serum can be a useful marker for the diagnosis, monitoring and followup treatment of neuroblastoma patients.

It has been reported that an antibody from a melanoma patient recognized an autoantigenic ganglioside related to GD2, Watanabe et al., J. Exp. Med., J. Exp. Med., 156, 1884 (1982). That antibody reportedly reacted relatively specifically with neuroectodermal-derived tumors when tested by immune adherence assays. However, the study employing the human antibody failed to apply the more discriminating immunoperoxidase technique for staining of frozen normal and tumor tissues that also provides more useful information for clinical purposes, since it detects even small tumor infiltrates and metastases into normal organs. In addition, there is thus far no published report providing a complete description of the tissue distribution of the GD2 antigen in normal tissues.

Still further, the use of monoclonal antibodies for the treatment of cancer patients has been considered as a possible adjunct to conventional

treatment protocols. However, the choice of the appropriate monoclonal antibody for this purpose depends not only on the intrinsic properties of the antibody used but also on the characteristics and surface expression of its specific target antigen. The results discussed hereafter also show that monoclonal antibody Mab 14.18 directed against the tumor-associated ganglioside GD2 can be used as an immunotherapeutic agent for the treatment of some tumors.

The fact that the GD2 antigen was shown to be heavily expressed on most excised melanoma and SCCL tumors, as well as on numerous tumor cell lines, Pukel et al., J. Exp. Med., 155, 1133 (1982) and yet is virtually absent from most normal tissues, suggests that it might be a good target antigen for in vivo specific immunotherapy, and tumor imaging. The results discussed hereinafter demonstrate that Mab 14.18 can mediate effective in vitro cytolysis of human tumor cells in the presence of human complement, and that effector cells from normal individuals are able to specifically induce lysis of tumor target cells having ganglioside GD2 expressed thereon.

In a recent report by Kipps et al., J. Exp. Med., 161, 1 (1985), using isotype switch variants of a Mab directed to an epitope on Class I human histocompatibility antigens, an IgG2a isotype variant was shown to be more effective in directing ADCC than the corresponding IgG1 or an IgG2b variant. Park et al., Cellular Immunol., 84, 94 (1984), reported that a monoclonal antibody of IgG2b isotype could sensitize K562 human erythroleukemia cells to ADCC-mediated lysis. In this case, it was reported that the Mab accelerated killing of the target cells

by large granular lymphocytes known to be enriched in natural killer (NK) cells. Taken together, the results of these studies indicate that monoclonal antibodies may not only be useful reagents for the immunotherapy of cancer, but also that different Mabs can induce tumor killing by several different or even a combination of effector mechanisms.

Some additional recent reports suggest that mouse monoclonal antibodies are relatively well tolerated in humans and pose minimal risks and few, if any, side effects, Oldham et al., J. Clin. Oncol., 2, 1235 (1984). In using murine Mab 9.2.27, an IgG2a monoclonal, to treat melanoma patients, that antibody was shown by the above workers to localize specifically to the tumor site with little if any adverse side effects, but provided no apparent clinical improvement of the disease over the period of time of their study in stage four patients with large tumor burdens.

It has also been reported that intravenous injection of Mab R24 (IgG3) that is directed to ganglioside GD3, caused a marked tumor regression in 3 of 11 patients with substantial tumor burdens, Houghton et al., Proc. Natl. Acad. Sci. (USA), 82, 1242 (1985). In that paper, it was reported that Mab R24 localized in the tumor causing immediate local inflammation. Biopsies of those tumors revealed the infiltration of both complement and mononuclear cells. It was also reported in Dippold et al., Cancer Res., 44, 806 (1984), that Mab R24 could kill GD3-containing human melanoma cells in vitro after prolonged exposure (greater than 24 hours) to the antibody suggesting an additional, as yet undefined, mechanism of tumor cell killing.

These initial reports suggest that the effectiveness of such an immunotherapeutic approach can depend on the ability of the antibody to evoke both humoral and cell-mediated effector mechanisms.

The results discussed hereinafter illustrate the use of a murine monoclonal anti-GD2 antibody to mediate cytolysis of human tumor cells in vitro and in vivo. The fact that this paratopic molecule can induce cell-mediated (ADCC), as well as complement-dependent killing, of these tumor cells can be attributable to this particular IgG3 antibody but can also be due to the very nature and expression of its antigenic target on the tumor cell surface.

GD2, like all other gangliosides, has a ceramide moiety that is embedded in the cellular plasma membrane with its antigenic carbohydrate moiety exposed. Therefore, monoclonal antibody-mediated cytolytic effector mechanisms that involve close contact with the target cell's plasma membrane may be more effective than those mediated by Mabs targeted to antigens that are associated extrinsically with the plasma membrane.

It is also likely that Mabs that can fix complement as well as induce cell-mediated cytotoxicity can use these mechanisms synergistically to destroy tumor target cells. It has been reported that activated human lymphocytes express membrane-associated proteases capable of cleaving the third complement component resulting in C3b binding to antibodies on lymphocytes, leading ultimately to enhancement of ADCC, possibly by augmenting effector cell-target contact, Erdei et al., Scand. J. Immuno., 20, 125 (1984).

The results discussed hereinafter clearly demonstrate the efficacy of the anti-GD2 antibody Mab

14.18 for effective in vitro killing of human melanoma cells by two distinct mechanisms; i.e. complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytoxicity (ADCC).

Another clinical use of Mab 14.18 is indicated by results of recent clinical investigations that suggest that patients with state IV neuroblastoma benefit from high dose chemotherapy and radiotherapy followed by rescue with autologous bone marrow, Hedley et al., Exp. Haematol., 1, 360. Since Mab 14.18 is highly cytotoxic to tumor cells expressing GD2 in complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity assays, it is useful for clearing bone marrow of tumor cells in vitro in combination with complement or in combination with effector cells.

The data from several studies, discussed hereinafter, that were performed to assess the chemical nature of the antigen recognized by Mab 14.18 indicate that the antigen is a ganglioside. Those results show that the antigen is, in fact, the disialoganglioside GD2 (disialosyl-N-triglycosyl ceramide).

Specifically, gangliosides separated by one-dimensional thin layer chromatography, when reacted with Mab 14.18, and then with an indicating means showed immunostaining of a single ganglioside component that comigrated with purified GD2. In addition, gangliosides extracted from various human tumor cell lines that were resolved on one-dimensional thin layer chromatography also showed immunostaining with Mab 14.18 of a material that comigated with purified GD2 standards. These same tumor cells react with Mab 14.18 in ELISA assays, and on that basis, a whole panel of tumor cells are

believed to have significant amounts of GD2 expressed thereon as shown in Table 1 hereinafter. It is also believed that the tumor cell lines and tissues that did not react with Mab 14.18 in studies whose results are shown in Tables 1 and 2 hereinafter, do not contain sufficient amounts of the ganglioside GD2 expressed on their cell membrane surfaces to be detected by these methods.

The results discussed below were obtained using the Mab 14.18 on numerous select types of tumor cells and tissues expressing ganglioside GD2 thereon. It is to be understood, however, that the results discussed hereinbelow are illustrative of embodiments utilizing only a few cell types and the present invention is not intended to be so limited but to further include other cell types having ganglioside GD2 expressed thereon.

II. RESULTS

A. Detection of GD2 on The Surfaces Of Tumor Cells

1. Identity of Antigen Recognized

To demonstrate the identity of the antigen to which Mab 14.18 specifically binds, gangliosides were directly visualized on TLC plates using various ganglioside-specific antibodies. The TLC plates contained total ganglioside extracts from human small cell carcinoma (SCCL) and human large cell lung carcinoma cell lines, as well as authentic GD2 and GD3 standards. The gangliosides were separated by TLC and then subjected to staining with either resdorcinol or antibodies.

As shown in FIGURE 1, the total ganglioside profile of NCI H-69 SCCL cells (panel A) and NCI H-82H SCCL cells (panel B) as visualized by resorcinol indicates the presence of all the major

ganglioside species and includes a doublet that comigrates with authentic GD2. This GD2 doublet could be identified in NCI H-69 and NCI H-82 SCCL cell lines as GD2 by immunostaining (FIGURE 2) with either Mab 14.18 (lanes A & B) or Mab 126 (lanes D & E). In contrast, gangliosides extracted from large cell lung carcinoma cells, NCI H-460 (lanes C & F), did not show reactivity with either of the anti-GD2 monoclonal antibodies. Anti-GM2 antibody, however, did stain corresponding doublets present in the gangliosides extracted from all three cell lines (lanes G,H and I). These data show that Mab 14.18 reacts specifically with the ganglioside GD2.

2. ELISA Reactivity of Monoclonal Antibody 14.18 with Different Tumor Cell Lines

The ability of Mab 14.18 to bind to various cells was screened using twenty-four different cell lines. Binding of Mab 14.18 to those cells was determined by a standard enzyme-linked immunoabsorbent assay (ELISA technique) discussed in detail hereinafter in Section III. The binding reaction pattern obtained is shown in Table 1 below:

TABLE 1

REACTIVITY OF MABS WITH HUMAN CULTURED CELLS[1]

| Cells Screened[2] | Mab (Antigen bound)[3] 14.18 (GD2) | 126 (GD2) | 5-3 (GM2) | 11C64 (GD3) | W6/32 Class I HLA |
|---|---|---|---|---|---|
| LUNG TUMORS | | | | | |
| Small Cell | | | | | |
| T293 | ++++ | ++++ | ± | - | ++ |
| NCI H-69 | +++ | ++ | ± | - | - |
| NCI H-82 | +++ | ++ | ++ | - | - |
| NCI N-47 | ++++ | +++ | ++ | - | - |
| Squamous Cell | | | | | |
| USCSL-1 | - | - | + | ± | +++ |
| SK-Mes-1 | - | - | ++ | - | +++ |
| CALU-6 | - | - | + | - | ± |
| Adenocarcinoma | | | | | |
| T291 | ++ | ++ | ++ | - | +++ |
| UCLA-P3 | - | - | ± | - | - |
| Large Cell | | | | | |
| NCI-H460 | ± | ± | ++ | - | +++ |
| OTHER TUMORS | | | | | |
| Melanoma | | | | | |
| M21 | ++++ | ++++ | +++ | ++ | +++ |
| Melur | ++ | +++ | ++ | ++++ | ++++ |
| M14 | ++++ | ++++ | ++ | ++++ | +++ |

| | | | | | |
|---|---|---|---|---|---|
| Neuroblastoma | | | | | |
| LAN-5 | ++++ | ++++ | ± | ± | - |
| LAN-1 | ++++ | ++++ | ND | - | +++ |
| SK-NAS | ++++ | ++++ | ND | - | +++ |
| Glioblastoma | | | | | |
| U87MG | + | ++ | + | - | ± |
| U138MG | ++++ | ++++ | + | - | - |
| U373MG | + | ++ | + | - | ++ |
| Pancreatic | | | | | |
| COLO 357 | - | - | +++ | ± | ++++ |
| SG | - | - | - | - | - |
| FG | - | - | - | - | + |
| Leukemic | | | | | |
| Molt-4 | - | - | +++ | - | - |
| Monocytic | | | | | |
| U937 | - | - | - | - | ++ |

[1] OD 492 nm reactivity determined by ELISA and described in Materials and Methods is designated as follows: - =0.00-0.049; ± =0.050-0.100; + =0.101-0.249; ++ =0.250-0.499; +++ =0.500-0.999; ++++ =1.000 or more.

[2] Sources of cell lines are discussed in Materials and Methods, Section III.

[3] Sources of Mabs are discussed in Materials and Methods, Section III.

As can be seen in Table 1, all cell lines derived from melanoma, neuroblastoma and small cell lung carcinoma that were used in this study exhibited strongly positive binding by (reaction with) Mab 14.18. The glioma cell line U138MG was also strongly positive, whereas two other glioblastoma cell lines bound very weakly. Futhermore, Mab 14.18 exhibitecd moderate binding with one lung adenocarcinoma (T291) but not another (UCLA-P3), and very weak binding with the large cell lung carcinoma cell line, H-460. Mab 14-18 did not bind to cell lines derived from numerous other tumor types including squamous skin carcinoma, pancreatic carcinoma and T-cell or monocytic leukemias. These data indicate that the antigen recognized and bound by Mab 14.18 is preferentially expressed on cell lines derived from neuroectodermal tumors or from small cell lung carcinoma, which itself has been postulated to be of neuroectodermal origin.

In comparing the reactivities of Mabs 14.18 and 126, both of which bind GD2, it is noted that Mab 14.18 exhibits somewhat better binding to two SCCL (NCI H-69 and H-82) than does Mab 126. It is also noted that Mab 14.18 binds more poorly to one melanoma cell line (Melur) and two glioblastoma lines (U87MG and U373MG) than does Mab 126. These results illustrate the narrowed binding specificity of Mab 14.18 as compared to other monoclonals, such as Mab 126.

Panel B of FIGURE 3 illustrates typical immunoperoxidase staining observed when Mab 14.18 was admixed with and bound to a human tissue containing small cell lung carcinoma cells. For comparison, another section of that tissue sample (Panel A) was stained with hematoxylin plus eosin. These panels of

Figure 3 clearly depict the very strong staining obtained by Mab 14.18 with all tumor cells of the tissue section shown.

It is of interest that at the time the above binding study was carried out, each of the particular tumors had been frozen and stored. The strongly positive staining obtained therefore indicates that the antigen recognized by Mab 14.18 was not denatured by freezing or by storage.

3. Reactivity of Mab 14.18 With Different Frozen Normal and Malignant Tissues

The ability of Mab 14.18 to bind to a relatively large number of frozen normal and malignant tissues was also screened. Binding was assayed by an immunoperoxidase technique discussed hereinafter in the Materials and Methods, Section III, using frozen human tissues obtained at autopsy.

Ten out of thirteen melanoma tissues and three out of six small cell lung carcinoma tissues were found to show significant positive binding. The proportion of autopsy sample originally expressing GD2 *in vivo* may be higher than that observed in this study due to a possible degradation of antigen that can occur post mortem.

Among normal tissues, there was weak reactivity of Mab 14.18 with all splenic red and white pulp, 40% of lung stroma, 10% of skin and marginal reactivity with 50% of kidney stroma. All the other normal tissues assayed were negative. The improved specificity exhibited by Mab 14.18 towards non-tumor cells as compared to that exhibited by Mab 126 that also binds to ganglioside GD2 is further illustrated in Table 2, below.

TABLE 2

TISSUE REACTIVITY OF ANTI-GD2 MABS BY IMMUNOPEROXIDASE ASSAYS[1]

| Tissues Screened[2] | 14.18 (IgG3) [anti-GD2] | 126 (IgM) [anti-GD2] | 11C64 (IgG3) [anti-GD3] |
|---|---|---|---|
| **Malignant Tissues** | | | |
| SCCL | 3/6,3+—2+ | 3/6,3+ | ND |
| Melanoma | 10/13,3+ | 9/13,3+ | 12/15,3+ |
| **Normal Tissues** | | | |
| Skin | 1/9,1+ | 4/9,±s | 5/6,1+ |
| Lung | 4/10,1+s | 3/10,1+s | 1/2,± |
| Colon | 0/2 | 0/2 | 2/2,1+ |
| Duodenum | 0/2 | 2/2,1+ | 1/2,± |
| Jejunum | 0/2 | 0/2 | 2/2,± |
| Kidney | 1/2,±s | 2/2,±s | 2/2,1+ |
| Liver | 0/2 | 1/2,±s | 2/2,1+ |
| Ovary | 0/2 | 0/2 | 0/2 |
| Pancreas | 0/2 | 2/2,±s | 2/2,1+ |
| Spleen | 2/2,1+r,w | 2/2,±r | 1/2,± |
| Testes | 0/2 | 0/2 | 1/2,1+ |

[1] Positive-reacting samples of total sample ( / ). Relative strength of reaction was: 3+, strong positive reaction; 2+, moderate positive, 1+, weak positive reaction; ±, marginal reaction. Tissues reacting were: s, stroma; r, splenic red pulp; w, splenic white pulp; ND, not determined.

[2] Tissue sections from human subjects taken at autopsy and thereafter frozen.

4. Assay Methods and Systems

The above and previously-discussed results demonstrate that a monoclonal paratopic molecule of the present invention present as an intact antibody reacts with and binds to ganglioside GD2. A particular hybridoma used in producing (secreting) a monoclonal paratopic molecule of the present invention (Mab 14.18) was deposited pursuant to the Budapest Treaty on June 4, 1986 in the American Type Culture Collection of Rockville, MD, and bears the designation ATCC HB 9118.

The previously discussed results also demonstrate that a diagnostic method of the present invention that utilizes the monoclonal paratopic molecule of the present invention is useful for assaying for the presence of ganglioside GD2 on a variety of tumor cells.

As described in greater detail hereinafter, the present invention thus provides several methods, in the solid phase and liquid homogeneous phase as well as in vivo and in vitro, for assaying for the presence of ganglioside GD2 in a sample. Each of those methods utilizes the following steps: (a) admixing and contacting a sample to be assayed with an aqueous composition containing an effective amount of a monoclonal paratopic molecule of this invention; (b) maintaining that contact under biological assay conditions for a predetermined time period sufficient for the paratopic molecule to immunoreact with any ganglioside GD2 present in the sample to form an immunocomplex; and (c) determining the presence of paratopic molecule that immunoreacted with ganglioside GD2.

Exemplary methods and diagnostic systems containing one or more reagents useful in carrying out those methods are described hereinafter.

(a) Imaging

An in vivo method for locating cells such as neuroectodermal tumor cells having ganglioside GD2 expressed thereon using an imaging technique is contemplated in one aspect of the invention. In such a method, a monoclonal paratopic molecule of the present invention is labeled with an indicator labelling means or group. The indicating group or label is utilized in conjunction with the monoclonal paratopic molecule as a means for determining that ganglioside GD2 has bound to the antibody.

When a monoclonal paratopic molecule of this invention is utilized for the in vivo imaging of tumors, as discussed below, it is preferred that the paratopic polypeptide portions such as Fab and $F(ab')_2$ portions be used rather than whole, intact antibodies. The reason for this preference stems principally from the fact that the presence of Fc antibody portions from an animal species different from the animal whose tumor is to be imaged can lead to subsequent immunological complications, although, as noted previously, intial investigations indicate the presence of substantially no such complications.

Radioactive elements provide a class of label that is particularly useful in in vivo imaging, although also useful in in vitro assays as are discussed hereinafter. An exemplary radiolabelling agent that can be utilized in the invention is a radioactive element that produces gamma ray emissions. Elements that themselves emit gamma rays such as $^{125}I$ represent one class of gamma ray emission-producing radioactive element indicating groups. Another class of useful indicating groups are those elements such as $^{11}C$, $^{18}F$, $^{15}O$ and $^{13}N$ which themselves emit positrons. The positrons

so emitted produce gamma rays upon encounters with electrons present in the animal's body.

A radioactive monoclonal paratopic molecule can typically be made by isolating the monoclonal antibody and then labelling the monoclonal antibody with one of the above or another appropriate radioactive elements as described in U.S. Patent No. 4,381,292.

The radiolabeled paratopic molecule such as Mab 14.18 or the paratope-containing portion thereof is then introduced as by injection into the blood stream of an animal having a tumor that exhibits ganglioside GD2 on its surface. The labeled paratopic molecule forms a complex with the ganglioside GD2 on the tumor cell surface, and after a suitable, predetermined time, such as about 18 to about 24 hours to permit clearance of unbound labeled paratopic molecule from the body, the animal or a portion of the animal is scanned.

The animal is scanned with a gamma ray emission counting machine such as the axial tomographic scanner commercially available under the designation CT (80-800 CT/T) from General Electric Company (Milwaukee, WI), or with a positron emission transaxial tomography scanner such as that designated Pett VI located at Brookhaven National Laboratory. Such scanning can provide an image of the tumor as well as information as to the location, size and shape of the tumor because of the specificity of the radiolabeled paratopic molecule utilized.

In another embodiment, a monoclonal paratopic molecule is labeled with an indicating group containing an element that is active in nuclear magnetic resonance (NMR) spectroscopy; i.e., an NMR-active element. Many such elements are

commercially available in useful form and are exemplified by $^{1}H$, $^{13}C$, $^{15}N$, $^{19}F$ and the like.

It is particularly preferred to utilize an indicating group containing the NMR-active $^{19}F$ atom, or a plurality of such atoms inasmuch as (i) substantially all of naturally abundant fluorine atoms are the $^{19}F$ isotope and thus substantially all fluorine-containing compounds are NMR-active; (ii) many chemically active polyfluorinated compounds such as trifluoroacetic anhydride are commercially available at relatively low cost, and (iii) many fluorinated compounds have been found medically acceptable for use in humans such as the perfluorinated polyethers utilized to carry oxygen as hemoglobin replacements.

Another particular advantage of the use of fluorine-containing NMR-active indicating groups is that the body contains very little fluorine under normal conditions. Consequently, by using an NMR-active element such as fluorine that is otherwise substantially absent from the animal, background signals due to bodily fluorine atoms are substantially absent. Thus, the principal signals observed are due to the labeled paratopic molecule-ganglioside GD2 complex.

In this embodiment, a paratopic molecule such as an intact or substantially intact Mab 14.18 is preferably labeled with a fluorine-containing material such as trifluoroacetic anhydride or hexafluoroethanol to form a fluorinated amide or ester derivative, respectively. Thereafter, the fluorinated antibody is introduced as by injection into the bloodstream of the tumor-containing animal. After a predetermined amount of incubation time for the labeled antibody to complex with the ganglioside

GD2 on the tumor cell surface, a so-called "whole-body" NMR determination is carried out using an apparatus such as one of those described by Pykett, Scientific American, 246, 78-88 (1982) to locate and form an image of the tumor.

Thus, the above method of locating (imaging) ganglioside GD2-containing cells such as those of a neuroectodermal tumor in vivo in an animal includes the steps of:

(a) providing a composition containing a monoclonal paratopic molecule of the present invention bonded to an indicating group. Typical compositions include about 5 to about 1000 milligrams (mg) of the labeled antibody in an aqueous medium such as that provided by water alone, an aqueous saline, phosphate-buffered saline or other aqueous buffer solution per square meter ($m^2$) of recipient, and more preferably about 10 to about 20 $mg/m^2$. The amount of paratopic molecule utilized depends, inter alia, upon the animal, the tumor size and the isotype of paratopic molecule, where an intact antibody is used as paratopic molecule. The useful indicating groups include gamma ray emission-producing elements, NMR-active elements and the like.

(b) The composition so provided is introduced into the bloodstream of a recipient animal to be assayed for the presence of cells bearing ganglioside GD2 on their surfaces, as by injection.

(c) The animal so injected is maintained for a predetermined period of time sufficient for the indicating group-bonded paratopic molecule to form an immunecomplex on the surface of the ganglioside GD2-bearing cells, and preferably for non-bound, labeled paratopic molecule to clear from the recipient animal's body.

(d) The animal is then scanned with a means for detecting the location of the complexed indicating group, and thereby the location of the GD2-bearing cells. Typical detecting means include usually used gamma ray emission detectors, those machines used in positron emission tomography (PET) and so-called "whole body" NMR spectrometers which may in practice only scan a portion of the body at any time. The result of such scans can produce an image of the GD2-bearing cells such as a tumor after data processing by known techniques.

(b) In Vitro Assays

In vitro assays for the presence of ganglioside GD2 are particularly contemplated herein, and are discussed hereinafter. Such assays utilize a variety of indicating means in addition to the radioactive and NMR-active elements discussed before.

The indicator labelling means can be linked directly to a paratopic molecule of this invention, to a useful antigen, or can comprise a separate molecule. It is particularly preferred that the indicator means be a separate molecule such as antibodies that bind to a paratopic molecule of this invention such as goat or rabbit anti-mouse antibodies. Staphylococcus aureus protein A, sometimes referred to herein as protein A, can also be used as a separate molecule indicator or labelling means where an intact or substantially intact paratopic molecule of this invention is utilized; i.e., where a paratopic molecule containing the portion of the Fc region of Mab 14.18 that is bound by protein A is used. In such uses, the protein A itself contains a label such as a radioactive element discussed before or a fluorochrome dye, as is discussed hereinafter.

An exemplary indicator labelling means is a fluorescent labelling agent that can be chemically linked to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labelling agents are fluorochromes such as fluorescein isocyanate (FIC), flourescein isothiocyanate (FITC), dimethylamino-naphthalene-S-sulphonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine rhodamine B200 sulphonyl chloride (RB 200 SC) and the like. A description of immunofluorescence analysis techniques is found in DeLula, "Immunofluorescence Analysis", in Antibody As A Tool, Marchalonis et al., eds., John Wiley & Sons, Ltd., pp. 189-231 (1982), which is incorporated herein by reference.

The indicating group can also be a biologically active enzyme, such as horseradish peroxidase (HRP) or glucose oxidase, or the like. Where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a antibody-antigen complex has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

An enzyme-linked label can also be utilized with the enzymic label linked directly to a paratopic molecule of this invention or linked to a second moiety such as an antibody that immunoreacts with a paratopic molecule of this invention. The latter technique is specifically exemplified herein.

It is to be understood that useful label molecules or atoms linked to another molecule are operatively linked to each other. Thus, the function of the label is not substantially impaired by the linkage, nor is the function of the other molecule to which the label is linked substantially impaired by that linkage.

Several in vitro methods are available for detecting the presence of ganglioside GD2 in a sample to be assayed.

In one embodiment of the invention, a solid phase assay method is contemplated for detecting the presence of ganglioside GD2 in a sample to be assayed such as a ganglioside mixture separated by TLC, cells suspended in an aqueous medium such as PBS, or a body fluid such as plasma or serum. This method comprises the steps of: (a) providing a solid matrix on which to affix the assayed sample; (b) admixing and contacting an aliquot of a liquid sample (cell suspension, plasma, serum or the like) to be assayed with the solid matrix to form a solid-liquid phase admixture; (c) maintaining the admixture for a predetermined time (typically about 10 to about 24 hours) sufficient for the sample to affix to the matrix and form a solid phase support; (d) separating the solid and liquid phases; (e) admixing and contacting an aqueous composition containing an effective amount of a paratopic molecule of this invention with the separated solid phase to form a second solid-liquid phase admixture; (f) maintaining the second solid-liquid phase admixture under biological assay conditions for a predetermined time (typically about 0.5 to about 2 hours) sufficient for the paratopic molecule to react and form an immunocomplex with ganglioside GD2 present in the

sample; (g) separating the solid and liquid phases; and (h) determining the presence of ganglioside GD2 that immunocomplexed with the antibody.

The presence of the ganglioside GD2 that immunocomplexed with the paratopic molecule can be determined in a number of ways. In one preferred embodiment, that determination is made by the steps of (i) admixing and contacting a liquid solution containing an effective amount of indicator labelling means (such as described hereinabove) with the solid phase obtained after step (g) above to form a third solid-liquid phase admixture, the indicator labelling means providing a means of detecting the presence of the paratopic molecule that reacted with ganglioside GD2; (ii) maintaining the admixture under biological assay conditions for a predetermined time (typically about 0.5 to about 2 hours) sufficient for the indicator labelling means to react and form a complex with the paratopic molecule; (iii) separating the solid and liquid phases of the third solid-liquid phase admixture; and (iv) determining the presence of paratopic molecule that immunocomplexed with ganglioside GD2.

The results of such an assay method for a cell suspension are shown in Table I discussed hereinbefore.

In another embodiment of the invention, an assay method for detecting the presence of ganglioside GD2 in a tissue sample to be assayed is contemplated. The method comprises the steps of: (a) providing a solid phase support comprised of a tissue sample to be assayed affixed to a solid matrix such as a slide; (b) admixing and contacting the solid phase-containing sample with an aqueous composition containing an effective amount of a

paratopic molecule of the invention that binds to ganglioside GD2; (c) maintaining that contact under biological assay conditions for a predetermined time (typically about 0.5 to about 2 hours) sufficient for the paratopic molecule to react with ganglioside GD2 present in the sample to form an immunocomplex; and (e) determining the presence of paratopic molecule that reacted with ganglioside GD2.

The determination of the presence of paratopic molecule that bound to ganglioside GD2 in the tissue section sample is typically carried out by admixing and contacting the immunocomplex with an indicator labelling means and maintaining that contact under biological assay conditions for a predetermined time (typically about 0.5 to about 2 hours) sufficient for the indicator labelling means to react and form a complex with the antibody, the indicator labelling means providing a means of determining the presence of monoclonal paratopic molecule that reacted with ganglioside GD2. After the contact maintenance periods, the solid and liquid phases that result are typically separated as by washing to remove any unreacted reagents (e.g. paratopic molecule or labelling means).

The results from such an assay method are shown in FIGURE 3 and in Table II hereinbefore, as well as in the analysis of FIGURE 2 where the TLC plate provided the solid matrix. As noted earlier, since the present monoclonal paratopic molecule is of murine origin and the tissues studied in FIGURE 3 and Table II were human, the HRP-linked second antibody (goat anti-mouse) indicator labelling means utilized in the assays of Table II did not cause background interference by binding to human antibodies that may have been present in the samples studied.

Details for the above methods are given hereinafter wherein the indicator labelling means is horseradish peroxidase-labeled goat anti-mouse IgG + IgM. The aforementioned radiolabelling agents, fluorescent molecules and radioactive elements can also be utilized in the above method as indicator labelling means.

The in vivo imaging assay is a solid phase assay as are the in vitro solid phase assay methods described before, and those solid phase assays can be described generically. Each includes a solid support that includes an affixed sample to be assayed, e.g., a developed TLC plate, a tumor, a microtiter plate well and a mounted tissue section. The solid support is also admixed and contacted with an aqueous composition containing an effective amount of the paratopic molecule, whether that contacting is done by the blood of a recipient animal or from a buffer solution. The maintenance, binding determination and other aspects are also analogously present in each assay method.

In still another solid phase assay, an effective amount of a monoclonal paratopic molecule of this invention is affixed to the solid matrix to form the solid support. An aqueous composition of a sample to be assayed such as serum, plasma, a cell suspension or lysate is admixed and contacted with the solid support to form a solid-liquid phase admixture. That contact is maintained under biological assay conditions for a predetermined period of time sufficient for the affixed paratopic molecule to react with any ganglioside GD2 present in the sample to form an immunocomplex. The presence of paratopic molecule that reacted with ganglioside GD2 is then determined.

The presence and, if desired, amount of paratopic molecule bound to ganglioside GD2; i.e., immunocomplex formed, can be determined in a number of manners. In one embodiment, an excess of label-linked ganglioside GD2 is admixed with the solid phase that results from separating the support from the reaction mixture after the above maintenance step. The presence and amount of complex formed can thereafter be determined by comparison of the amount of labeled ganglioside GD2 that bound to a standard amount bound in a control study.

Thus, a solid phase method for assaying for the presence of ganglioside GD2 in a sample comprises the steps of: (a) providing a solid support comprising a solid matrix having affixed thereto either a sample to be assayed or an effective amount of a monoclonal paratopic molecule of this invention; (b) admixing and contacting that solid support with an aqueous composition containing either an effective amount of a paratopic molecule of this invention or a sample to be assayed, respectively, to form a solid-liquid phase admixture; (c) maintaining that contact under biological assay conditions for a predetermined period of time sufficient for the paratopic molecule to react with ganglioside GD2 present in the sample to form an immunocomplex; and (d) determining the presence of paratopic molecule that reacted with ganglioside GD2.

It is to be understood that liquid, homogeneous phase assay methods are also contemplated herein. Although determinations of the amount of binding between a monoclonal paratopic molecule and its antigen are sometimes difficult in homogeneous assays, such systems provide the advantage of not requiring a separation step and therefore are particularly amenable to automation.

When ultilized in a homogeneous, liquid assay, a monoclonal paratopic molecule of this invention is linked to an indicating means such as an enzyme, free radical or fluorochrome in a manner that binding (immunoreaction) of the paratopic molecule to its GD2 antigen changes, typically inhibiting or diminishing, the signal provided by the indicator. Exemplary systems utilizing such label-linked paratopic molecules are discussed in U.S. Patent No. 4,160,645 and in O'Sullivan et al, Ann. Clin. Biochem., 16, 221 (1979), whose disclosures are incorporated herein by reference.

A homogeneous, liquid assay for the presence of ganglioside GD2 in a sample, comprises the steps of: (a) admixing and contacting an aqueous, liquid sample such as plasma, serum or a cell suspension with an aqueous composition containing a predetermined amount of monoclonal paratopic molecule of this invention linked to an indicating means whose signal is changed when the paratopic molecule binds to its antigen; (b) maintaining that contact under biological assay conditions for a predetermined time period sufficient for the paratopic molecule to react with ganglioside GD2 present in the sample to form an immunocomplex; and (c) determining the presence of paratopic molecule that reacted with ganglioside GD2.

The presence of an immunocomplex in the above assay is typically carried out by comparing the signal produced from a known amount of the label-linked paratopic molecule in the absence of sample (control) to that produced in the presence of sample. Where the amounts of label-linked paratopic molecule used in the presence and absence of sample are the same, a difference in the signal provides a qualitative determination. Where those amounts are

different, standard curves are readily prepared to provide the desired comparative signal values from which the qualitative determination can be made.

Quantitative determinations can also be carried out with the above homogeneous, liquid assay method. For quantitation, standard curves are preferably prepared in which known amounts of the GD2-containing entity to be assayed are immunoreacted with the known amount of label-linked paratopic molecule to provide varying amounts of signal to be compared to assay results.

(c) Diagnostic Systems

The monoclonal paratopic molecule of the invention can also be utilized in a diagnostic system typically in kit form for assaying for the presence of human neuroectodermal, SCCL or other tumors or cells having ganglioside GD2 expressed thereon, as well as liquid compositions such as serum that contain the ganglioside. The system includes in at least one container as an active ingredient, an effective amount of the monoclonal paratopic molecule of the invention in dry, solution or dispersion form. The system also contains an indicating means such as those described before that when introduced into a sample, binds selectively with the monoclonal paratopic molecule. Alternatively, the indicating means can also be linked directly to the paratopic molecule active ingredient.

The diagnostic system can also include a solid matrix such as 96-well microtiter plates sold under the designation Falcon Microtest III Flexible Assay Plates (Falcon Plastics, Oxnard, CA) or a microtiter strip containing twelve wells in a row, such as those strips sold under the designation Immulon I and II (Dynatech, Alexandria, VA). The

microtiter strip or plate is made of a clear plastic material, preferably polyvinyl chloride or polystyrene. Alternative solid matrices for use in the diagnostic system and a before-described method of this invention include polystyrene beads, about 1 micron to about 5 millimeters in diameter, available from Abbott Laboratories, North Chicago, IL; polystyrene tubes, sticks or paddles of any convenient size; and polystyrene latex whose polystyrene particles are of a size of about 1 micron and can be centrifugally separated from the remainder of the latex.

The solid matrix can also be made of a variety of materials such as cross-linked dextran, e.g. Sephadex G-25, -50, -100, -200 and the like available from Pharmacia Fine Chemicals of Piscataway, New Jersey, agarose and cross-linked agarose, e.g. Sepharose 6B, CL6B, 4B, CL46 and the like also available from Pharmacia Fine Chemicals.

The diagnostic system can further include a standard against which to compare the assay results and various buffers in dry or liquid form for, inter alia, washing the wells, diluting the sample or diluting the labeled reagent.

B. Cytotoxicity of Mab 14.18 to Tumor Cells Expressing Ganglioside GD2

The capacity of Mab 14.18 to mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) in vitro were assessed since the cytotoxicity against tumor cells mediated by monoclonal antibodies can be important for clinical applications of such antibodies.

1. Complement-Dependent Cytotoxicity (CDC)

In an in vitro CDC assay using about $1x10^4$ cells/well of $^{51}Cr$-labeled target cells, whole Mab 14.18 at a concentration of about 20 micrograms per well in an aqueous medium of 75 microliters to which 150 microliters of fresh human serum (diluted 1:3 in PBS) as a source of complement proved to be highly efficient in mediating the killing of T293 human SCCL cells. As shown in FIGURE 4, Mab 14.18, which strongly fixes complement, killed over 90 percent of these tumor cells within a time period of about one hour in the presence of complement. Without complement or with complement but in the presence of a non-binding antibody, the cytotoxicity against these target was not significant.

The above results demonstrate that tumor cells having ganglioside GD2 expressed thereon are effectively killed in vitro by contacting those cells in the presence of exogenously supplied complement with a composition of the present invention comprising an effective amount of Mab 14.18 in a physiologically tolerable diluent. In vivo complement-dependent killing of tumor cells that express GD2 on their surfaces can be carried out similarly to that described above, with the complement being supplied endogenously by the recipient's body.

2. Antibody Dependent Cellular Cytotoxicity (ADCC)

To determine whether intact monoclonal antibodies directed to gangliosides could induce ADCC in conjunction with human effector cells, the T293 small cell lung carcinoma cell line was labeled with $^{51}Cr$. The resulting labeled cells were admixed at

$5x10^3$ cells/well with and maintained (incubated) in the presence or absence of Mab 14.18 at about 5 micrograms per well as well as mononuclear effector cells obtained from the peripheral blood of normal individuals in a total volume of 225 microliters.

As shown in FIGURE 5, effector cells from two normal donor individuals were able to specifically lyse between 20 to 40 percent of the GD2-containing small cell lung carcinoma target cell line depending on an effector to target cell ratio of between 100 to 200. Lysis of target cells in the absence of antibody is also shown in FIGURE 5 and is referred to as natural killer (NK) lysis. Depending on the effector to target cell ratio, the NK lysis remained very low; i.e., between 2 and 8 percent, and those low values were subtracted out in making the lysis calculations.

Similar studies were carried out using M21 melanoma cells, which express both GD2 and GD3 on their surfaces, and SK-NAS neuroblastoma cells, which express GD2 but not GD3 on their surfaces, as target cells. At effector to target cell ratios of 100, 50 and 25, Mab 14.18 induced 47, 19 and 7 percent lysis, respectively, in the 4-hour assay against M21 cells. The results against SK-NAS cells were 29, 8 and 4 percent lysis, respectively, at the 100, 50 and 25 effector:target cell ratios. Again, lysis due to the effector cells alone was taken into consideration in the above-calculated lysis percentages.

These results show that a composition containing an effective amount of Mab 14.18 as an active ingredient in a physiologically tolerable diluent can mediate ADCC in the presence of effector cells such as peripheral blood mononuclear cells that can be established <u>in vitro</u> by admixture at the

indicated ratio of effector to target cells. Because of this ability to kill tumor cells that have ganglioside GD2 expressed thereon, paratopic molecule 14.18 is also useful in vivo where recipient animals can provide the effector cells such as mononuclear cells in circulation necessary for Mab 14.18 to mediate ADCC of tumors expressing GD2.

Additional effector cells from an immunologically compatible donor can also be used to supplement the recipient's effector cells. Exemplary compatible donors include the recipient from whom effector cells can be obtained, kept in culture, and then provided back to the recipient as by injection along with Mab 14.18. Other suitable donors are those whose effector cells do not cause an adverse immunological response in the recipient as can be determined by standard procedures. Thus, the additional effector cells can be supplied ex vivo from an exogenous source.

The term "effector" cells as used herein refers to cells that are capable of mediating cellular cytotoxicity in the presence of specific antibodies. This cytotoxicity is dependent upon antibody and is therefore termed "antibody-dependent cellular cytotoxicity" (ADCC). Exemplary of such effector cells are human peripheral blood mononuclear cell populations such as are purified from heparinized blood by the well known method of gradient centrifugation over Ficoll-Hypaque.

The above results demonstrate that the paratopic molecule of the present invention, which is produced by the hybridoma having ATCC accession number HB 9118 and reacts and binds with ganglioside GD2 on the surface of human tumor cells, is useful to mediate killing of tumor cells having ganglioside GD2

expressed thereon by either or both of the CDC and ADCC mechanisms.

A monoclonal paratopic molecule of the present invention, particularly intact Mab 14.18, when present as an active ingredient in a cytotoxic amount dispersed in an aqueous physiologically tolerable diluent as discussed hereinafter, is therefore suitable for use as a composition for treatment of animals with tumors having GD2 expressed thereon. That composition can treat tumors via one or both of the before-discussed CDC and ADCC pathways. Thus, the administration of Mab 14.18 can provide a direct, anti-tumor cytotoxic effect.

A composition containing the monoclonal antibody Mab 14.18 as an active ingredient is typically administered in a unit dose having a cytotoxic amount of intact Mab 14.18 antibodies dispersed in an aqueous physiologically tolerable diluent such as serum, normal saline, water, Ringer's solution, lactated Ringer's solution, or phosphate-buffered saline. A cytotoxic amount of Mab 14.18 varies, inter alia, depending on the particular tumor cells treated and the amount of those cells.

For in vivo cytotoxicity generally about 5 to about 1000 milligrams (mg) of Mab 14.18 per square meter ($m^2$) of recipient animal surface area is useful, and more preferably, aobut 10 to about 150 $mg/m^2$ are used. Since an average adult human has a surface area of about 1.7 $m^2$, these unit doses can also be expressed as about 15 to about 250 mg per person. In such in vivo work, cytotoxic amounts of complement and/or effector cells are provided by the recipient's own body, and therfore complement and/or effector cells are present in physiological amounts. The effector cells can also be provided by the ex vivo exogenous route discussed before.

For in vitro cytotoxicity, a cytotoxic amount of Mab 14.18 is about 1 to about 200 micrograms per milliliter (mg/ml), and more preferably about 10 to about 100 mg/ml where about $0.5x10^4$ to about $10x10^4$ tumor cells per milliliter (cells/ml), and more preferably about $1x10^4$ to about $5x10^4$ tumor cells/ml, are utilized as targets for cytotoxicity. Here, cytotoxic amounts of complement and/or effector cells are supplied exogenously to the tumor cell culture.

A cytotoxic amount of complement can be provided by about 50 to about 500 microliters of fresh human serum, and more preferably about 100 to about 300 microliters of such serum per milliliter of a before-described Mab 14.18-target cell preparation. Effector cells are provided in a cytotoxic amount at an effector:target tumor cell ratio of about 25:1 to about 500:1, and more preferably at a ratio of about 100:1 to about 200:1.

The term "unit dose" refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

An above-described monoclonal paratopic molecule-containing composition can additionally include a cytotoxic agent linked to the monoclonal paratopic molecule of this invention. The cytotoxic agent acts to kill the tumor cells in addition to any action provided by the paratopic molecule. Thus, the specificity of the antibody combining site for the locating and binding to tumor cells having ganglioside GD2 expressed thereon is again utilized. The cytotoxic agent is operatively linked to the

paratopic molecule such that neither the cytotoxicity of the drug nor the binding ability of the paratopic molecule is substantially imparied by the linkage.

The cytotoxic agent utilized can be a drug such as adriamycin, the G-418 variant of neomycin, toxin molecules of plant or bacterial origin (immunotoxins, such as the bacterium Corynebacterium diphtheriae (diptheria toxin) and the seeds of plants Abrus precatorius (abrin) and Ricinis communis (ricin), or a mammalian toxin such as tumor necrosis factor (TNF), or the like. It is particularly preferred that the cytotoxic agent act at least in part by contact with cell membranes.

Adriamycin is exemplary of a particularly preferred cytotoxic agent. The adriamycin is bonded to a paratopic molecule through the adriamycin sugar ring-amino group via a dialdehyde such as glutaraldehyde, by a water-soluble carbodiimide or by other well known linking means.

Tritton et al., Science, 217, 248-249 (1982) reported linking of adriamycin to agarose beads. Those authors bonded the drug to the agarose beads as a means of contacting tumor cell membranes while avoiding general dissemination of the drug throughout the body tissues since adriamycin is known to have toxic effects upon the hearts of cancer patients. The cancer cells so contacted by Tritton et al. died.

It was suggested in an article appearing on page 69 of Science News, July 31, 1982 that a means other than the agarose beads would be needed for in vivo use on human patients. However, that suggestion was limited to one specific material, plasma membrane proteins, and did not include the use of the specific monoclonal paratopic molecules of the present invention inasmuch as monoclonal paratopic molecules

such as those disclosed herein have not heretofore been available.

Another preferred cytotoxic agent is a methotrexate conjugate attached to the monoclonal paratopic molecule of the present invention as by a carbodiimide linker at a ratio of 10 to 15 moles of drug per mole of paratopic molecule.

III. MATERIALS AND METHODS

A. Cell Lines

The following cell lines were used for screening of the monoclonal hybridoma antibody of the present invention; sources for each line are parenthesized following the cell line designation: small cell lung carcinoma (SCCL): T293 (Drs. Masie and Sato, University of California at San Diego, hereinafter UCSD); NCI H-69 (ATCC HTB 119), NCI H-82, and NCI N-417 (Drs. Minna and Gadzar, NIH, Bethesda, MD.); squamous cell carcinoma: USCSL-1 (Dr. Kan-Mitchel, University of Southern California); CALU-6, and SK-MES-1 (ATCC HTB 56, and ATCC HTB 58, respectively); adenocarcinoma of lung: T291 (UCSD), and UCLA-P3 (Dr. Morton, University of California at Los Angeles, hereinafter UCLA); large cell lung carcinoma: NCI-H-460 (Drs. Minna and Gadzar, NIH, Bethesda, Md.); melanoma: M14 and M21 (Dr. Morton, UCLA): Melur (Dr. U. Kodovsky, Dusseldorf, West Germany); neuroblastoma: LAN-1, LAN-5 (Dr. Seeger, UCLA) and SK-NAS (Dr. Helson, Memorial Hospital, NY); glioblastoma: U87MG, U138MG, and U373MG (ATCC HTB 14, ATCC HTB 16, and ATCC HTB 17, respectively); pancreatic carcinoma: COLO 357, FG, and SG (Dr. Kajiji, Scripps Clinic, La Jolla, CA); leukemia: Molt-4 (ATCC CRL 1582); and monocytic lymphoma: U-937 (ATCC CRL 1593).

All the hybridoma cell lines, and the tumor cell line M14, were grown in DMEM (MA Bioproducts, Walkersville, MD), supplemented with 10 percent fetal calf serum (Hyclone Laboratories, Logan, UT), 2 millimolar glutamine (Gibco Laboratories, Lawrence, MA), and 50 micrograms per milliliter gentamycin (MA Bioproducts, Walkersville, MD) at 37 degrees C in an atmosphere containing 7.5 percent $CO_2$. All the other tumor cell lines were grown in RPMI 1640 (Gibco) supplemented as above.

B. Monoclonal Antibody 14.18

Monoclonal antibody (Mab) 14.18 was prepared by immunization with neuroblastoma cell line LAN-1 using the standard hybridoma technology of Kohler et al., Nature, 256, 495 (1975). Briefly, BALB/c mice were immunized by injection once every week for a total of 4 weeks with $5x10^6$ LAN-1 neuroblastoma cells. Their splenocytes were removed, a suspension of the spenocytes was made, and the spenocytes were fused three days after the last injection with a non-secreting murine myeloma cell line Sp2/O subclone in the presence of a cell fusion promoter (polyethylene glycol 2000) to form hybridomas. Hybridoma 14.18 was selected by appropriate binding properties and growth in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 percent gamma globulin-depleted horse serum (Biocell Laboratories, Carson, CA), hypoxanthine, aminopterin and thymidine (HAT), medium that does not support growth of the unfused myeloma cells, and was subcloned using limiting dilution and culturing in separate containers. The resulting supernatant in each container was evaluated for the presence of the isotype of the Mab 14.18 as described hereinafter. The desired hybridoma was selected and cloned, and

Mab 14.18 was recovered from the supernatant above the clones.

Alternatively, monoclonal paratopic molecule 14.18 can be produced by introducing, as by injection, the hybridoma into the peritoneal cavity of a mammal such as a mouse. Preferably syngenic or semi-syngenic mammals such as mice are used, as in U.S. Patent 4,361,549, whose illustrative teachings are incorporated herein by reference. The hybridoma introduction causes formation of antibody-producing hybridomas after a suitable period of growth, e.g. 1-2 weeks, and results in a high concentration of the antibody being produced that can be recovered from the bloodstream and peritoneal exudate (ascites tumor fluid) of the host mouse. Although these host mice also have normal antibodies in their blood and ascites, the concentration of normal antibody is only about five percent that of the monoclonal antibody concentration.

The monoclonal antibody present in hybridoma supernatants is used without purification or can be recovered from the ascites or serum of the mouse using standard techniques such as affinity chromatography using an immunosorbant such as Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO), followed by elution from the immunosorbant using an acidic buffer such as sodium citrate at a pH value of about 4.6. The elution is monitored by optical density (OD) at 280 nanometers (nm), the protein-containing peaks are pooled, and the Mab-containing pool is dialyzed against PBS.

In addition, the following Mabs used in these studies are listed with their respective isotypes and sources denoted in parentheses: Mab 11C64 [IgG3 Reisfeld et al; Scripps Clinic and

Research Foundation, La Jolla, CA, hereinafter Scripps], directed against GD3 [Cheresh et al., J. Cell. Biol., 102, 688 (1986)]; Mab 9.2.27 (IgG2a, Reisfeld et al., Scripps) directed against chondroitin sulfate proteoglycan of human melanoma cells [Cheresh et al., J. Cell. Biol., 102, 688 (1986); Harper et al., J. Immunol., 132, 2096 (1984); and Bumol et al., Proc. Natl. Acad. Sci. USA, 79, 1245 (1982)]; and Mab 126 (IgM, Reisfeld et al., Scripps), directed against GD2 [Cheresh et al., J. Cell. Biol., 102, 688 (1986)]; W6/32 (IgG2a, Dr. P. Parham, Stanford University, Palo Alto, Ca.), recognizing the common structure of HLA Antigens [Parham et al., J. Immunol., 123, 342 (1979)]; and Mab 5-3 (IgM, Dr. P. Livingston, Sloan-Kettering Cancer Center,), directed against GM2.

Isotypes were determined by ELISA essentially as described hereinbelow in Section E with the following modifications. Fifty microliter volumes (1:1000 dilutions in PBS) of rabbit anti-mouse IgG2, IgG3 or IgM (Southern Biotech Associates, Birmingham, AL) were dried down per well of microtiter plate (Dynatech, Alexandria, VA). Hybridoma culture supernates diluted 1:2 in PBS were then added at fifty microliters per well and maintained at 4 degrees C for 1 hour. After 2 washes, fifty microliters of horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Biorad Laboratories, Richmond, CA) diluted 1:1000 were added to each well and maintained at 4 degrees C for 1 hour. ELISAs were then developed using o-phenylenediamine substrate as described in Section E.

C. Tissue Samples

Portions of fresh normal and malignant melanoma tissues were obtaned from the Surgical

Pathology Department of the Ida M. Green Hospital of Scripps Clinic, La Jolla, CA. SCCL tissues were obtained at autopsy and provided by Drs. D. Caslo and M. Chiapetta (Hybritech Inc., La Jolla, CA). Fresh tissue specimens were embedded in Tissue Tek-II O.C.T. (Miles, Naperville, IL), frozen in blocks using isopentane at liquid nitrogen temperature, and then stored at -70° C.

D. Immunoperoxidase Staining of Frozen Tissues

Sections of frozen tissue blocks, 4-6 microns thick, were cut on a microtome cryostat, mounted on glass slides, briefly air-dried, and either stained immediately or stored at -70 degrees C in airtight boxes. An indirect immunoperoxidase assay, similar to that described by Taylor, Path. Lab. Med., 102, 113 (1978), the teachings of which are incorporated herein by reference, was used to stain these slides.

Briefly, after washing in phosphate-buffered saline (PBS), pH 7.1, the sections were pre-incubated (contacted) for 15 minutes at room temperature in PBS containing 10 percent goat serum and 0.1 percent bovine serum albumin to block non-specific binding sites. Excess serum was then removed by aspiration, and Mab 14.18 supernatant diluted 1:2 in PBS was overlayed onto the sections to admix with and contact the tissues. That contact was maintained in a humid chamber for one hour, and was followed by a brief wash in PBS to remove unbound Mab 14.18. The washed tissues were thereafter contacted with a 1:50 dilution of horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (Bio-Rad Laboratories, Richmond, CA.) to bind the labeled goat anti-mouse antibody to Mab 14.18. This contact was maintained for a one

hour time period at room temperature, and was then followed by a rinse in PBS.

Admixture of the peroxidase-labeled sample with 0.6 milligrams/milliliter (mg/ml) of diaminobenzidine in 0.03 percent $H_2O_2$ provided a brown color in the area of the enzyme. After a rinse with PBS, the slides were washed in water and dehydrated in isopropyl alcohol. They were then cleared in xylene, mounted in Paramount (American Scientific Products, McGaw Park, IL), coverslipped and examined using an American Optical Microstar Series 20 microscope to determine which tissue areas, if any, were stained by the dye produced by the diaminobenzidine. For comparison to immunoperoxidase staining, frozen tissue slices were also stained using the well known hematoxylin plus eosin stain following usually used procedures.

E. Enzyme-Linked Immunosorbent Assay (ELISA)

Target cells to be assayed were washed and resuspended in PBS, and were then plated in flat-bottom polyvinyl chloride microtiter plates (Dynatech, Alexandria, Va.) at $5x10^4$ cells per well using 50 microliters of sample composition. The plates were then incubated overnight at 37 degrees C in a dry oven. The dried plates were stored at 4 degrees C until use. Prior to the ELISA assay, dried plates were rehydrated by two washes of two minutes each with 10 millimolar (mM) PBS, pH 7.4, containing 0.1 percent polyoxalkylene (20) sorbitan monolaurate (Tween 20) and 0.02 percent Thimerosal (sodium ethylmercurithiosalicylate; Sigma, St. Louis, MO.).

In order to reduce non-specific binding, hybridoma supernatants were diluted 1:2 in the above washing buffer containing 0.1 percent BSA as diluent. Fifty microliters of diluted hybridoma

supernatants were thereafter added to each well and incubated 1 hour at 4 degrees C on a gyroshaker to contact the Mab 14.18-containing supernatant with the assayed cells and to bind that paratopic molecule to ganglioside GD2. Following two washes of 2 minutes each, 50 microliters of peroxidase-labeled goat anti-mouse antibody (Bio-Rad, Richmond, CA), diluted 1:1000, were added to each well, and the reaction mixture was incubated at 4 degrees C for 1 hour to bind the labeled antibody to bound Mab 14.18.

The substrate used to assay bound peroxidase activity was prepared just prior to use and consisted of 400 microgram/ml o-phenylenediamine (Sigma, St. Louis, MO.) in 80 mM citrate-phosphate buffer, pH 6.0, containing 0.12 percent $H_2O_2$. After two final washes, 50 microliters of substrate solution were added to each well and color was allowed to develop for 15 minutes in the dark. Color development was stopped by adding 25 microliters of 4 molar (M) $H_2SO_4$ to each well, and the optical density at 492 nanometers (nm) was measured with a Multiskan ELISA Plate reader (Bio-Tek Instruments Inc., Burlington, VA)

F. Glycolipid and Ganglioside Preparation

A total lipid extract was prepared from malignant tissue culture cells, equivalent to 2 milliliters of packed cells, by homogenization in 40 milliliters (ml) of chloroform:methanol (2:1) for 5 minutes using mild sonication and vortexing, and was followed by filtration through a scintered glass filter. The residue was re-extracted with chloroform:methanol (1:1) and re-filtered. The combined filtrates were then subjected to rotary evaporation. The dried glycolipid extract was dissolved in 10 ml of chloroform:methanol, (2:1).

Gangliosides were partitioned into an aqueous phase as described by Ledeen et al., Methods in Enzymol., 83, 139 (1982), the teachings of which are incorporated herein by reference. This material was dialyzed exhaustively against cold distilled water and lyophilized.

The lyophilized crude ganglioside preparation was dissolved in 20 ml of methanol-chloroform-water (60:30:8), and applied slowly to a column (1 cm x 15 cm) of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ). The column was washed extensively with the above solvent and gangliosides were eluted with methanol-chloroform containing 0.8 M aqueous sodium acetate (60:30:8). Fractions were collected and assayed for the presence of gangliosides by thin layer chromography (TLC), as described hereinafter, using a variety of ganglioside standards. Fractions containing gangliosides were evaporated and dissolved in distilled $H_2O$ to be dialyzed and lyophilized.

The freeze-dried material was dissolved in chloroform:methanol (1:1) and applied to a column of Iatrobeads [poly(iso-butyl methacrylate) beads; Polyscience, Inc., Warrington, Pa.] as described by Leeden et al., Methods in Enzymol., 83, 139 (1982). The material eluted from this column was relatively free from contaminants.

G. Ganglioside Standards

Purified ganglioside standard GD2 was kindly supplied by Dr. R. K. Yu (Yale University, New Haven, CT.). Gangliosides GM3 and GM2 were supplied by Dr. J. Sudsmo (Scripps), and ganglioside GM1 was purchased from Supelco (Bellefonte, PA.). Purified ganglioside GD3 was a gift of Dr. S. Hakomore (Fred Hutchinson Cancer Research Center, Seattle, WA).

H. Thin Layer Chromography (TLC)

Silica gel plates (plastic backed, Merk and Co., Rahway, NJ) were activated by heating at 110 degrees C for 1 hour. Chloroform/methanol/0.2 percent aqueous $CaCl_2$ (55:45:10) was used for the development of the chromatograms. Samples were spotted 1.5 cm from the bottom of the TLC plates that were then placed in a developing tank presaturated with 100 ml of the above solvent. Chromatograms were developed for 1.5 hours at room temperature, after which the plates were allowed to dry. Appropriate lanes of chromatograms were cut and sprayed with resorcinol reagent to visualize gangliosides as described in Jourdian et al., J. Biol. Chem., 246, 430 (1971). Alternatively, the gangliosides were visualized by immunostaining with anti-ganglioside Mabs as described hereinbelow.

I. Immunostaining of Gangliosides Separated by TLC

The reactivity of anti-ganglioside Mabs with ganglioside separated by TLC was determined directly on the plates by using an immunostaining method originally described by Magnani, et al., Anal. Biochem., 109, 399 (1980). This procedure was modified by using an indirect ELISA detection system as described by Cheresh et al., J. Biol. Chem., 259, 7453 (1984).

Briefly, TLC strips upon which gangliosides had been separated were placed in separate, screw capped plastic culture tubes having a volume of prechilled PBS [0 degrees C] containing 1% polyvinylpyrrolidone (PBS-PVP) sufficient for immersion of the strips. The tubes were then agitated by rocking at 5 degrees C for 10 minutes to block non-specific binding sites. After decanting

the blocking solution, tubes were filled with appropriate Mab hybridoma culture supernates diluted 1:2 in blocking solution and maintained at 5 degrees C for 1 hour while undergoing rocking. The tubes were then decanted and successively washed three times in the same buffer (PBS-PVP) at 4 degrees C for 15 minutes per each wash. HRP-labeled goat anti-mouse antibody (Bio-Rad) diluted 1:1000 in the same buffer was then added, and the tubes were further maintained while rocking at 4 degress C for 1 hour. HRP-labeled antibody was detected using the same substrate system as for ELISA in Section E, except that the color reaction was stopped by decanting and drying instead of using sulfuric acid.

J. Antibody-Dependent Cellular Cytotoxicity (ADCC)

T293 SCCL cells were labeled with $^{51}Cr$ [sodium chromate at 1 millicurie/milliliter (mCi/ml), New England Nuclear, Boston, MA]. Routinely, $10^6$ cells were incubated with 200 microcuries $^{51}Cr$ for 2 hours at 37 degrees C in 1 ml of RPMI culture medium 1640 (Roswell Park Memorial Institute, Buffalo, NY) containing 10 percent fetal calf serum (FCS) (GIBCO Laboratories, Grand, Island, NY). The radiolabeled cells were washed two times with PBS, resuspended in RPMI 1640 and 10 percent (FCS), and were plated in 96-well round bottom tissue culture plates (Costar, Cambridge, MA) at $5x10^3$ cells/well in 10 microliters , and are hereinfter referred to as target cells.

The target cells so prepared were then treated with antibodies by admixture with about 5 micrograms per well of Protein A-Sepharose-purified Mab (200 micrograms/milliliter; 25 microliters) to contact the cells with the antibodies. Heparinized normal, healthy human peripheral blood mononuclear

cells (PBM) were purified over a Ficoll-Hypaque (Lymphoprep, Nyegaard & Co., Oslo, Norway) gradient and served as effector cells. The cells were then added to the Mab-treated target cells at the various target:effector cell ratios shown in FIGURE 5 in a final volume of 225 microliters. The microtiter plates were centrifuged in their entirety at 400xg for two minutes, and were then maintained by incubation at 37 degrees C in a humidified atmosphere of 7.5% $CO_2$ for 4 hours. Thereafter, the plates were centrifuged at 400xg for 5 minutes, 100 microliters of each supernate were removed, and the radioactivity determined using a Packard Gamma Counter (Packard Instrument Co. Inc., Downers Grove, IL).

The cell-mediated killing of the target cells was calculated as follows:

$$\text{percent lysis} = \frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum release cpm - spontaneous cpm}} \times 100$$

In the above equation, "spontaneous cpm" (counts per minute) represents the radioactivity released from target cells in a representative sample of the labeled cells in the absence of effector cells. "Maximum release cpm" was determined by treating a representative sample of the labeled target cells with the non-ionic detergent NP 40 [polyoxyethylene (9) octyl phenyl ether; Shell Oil Company] at a concentration of 1 percnet PBS. To calculate percent specific lysis attributable to ADCC, the percentage lysis due to effector cells in the absence of antibody (i.e., natural killer cell lysis) was subtracted from each value.

K. Complement-Dependent Cytotoxicity Assay (CDC)

Labeling of tumor target cells with $^{51}Cr$ was done as described for the ADCC assay except that

100 microCuries of $^{51}Cr$ were used and the incubation was for one hour. The cells were washed as described, and $10^4$ labeled cells were plated into each well of a 96-well microtiter culture plate that further contained Protein A-Sepharose-purified intact monoclonal antibody 14.18 at 20 micrograms per well and medium to a final volume of 75 microliters. After a 1 hour incubation at 37 degrees C, 150 microliters of fresh normal human serum (diluted 1:3 in PBS) as a source of complement were admixed, and the resulting culture was maintained by incubation at 37 degrees C for timed intervals. After such timed intervals the microtiter plate was centrifuged at 400xg for 5 minutes, 100 microliters of supernate were removed from each time interval sample, and the radioactivity was determined as for ADCC. The complement-dependent cytotoxicity of the monoclonal paratopic molecule was calculated as a percent of lysis as described before except that "spontaneous cpm" was determined from cultures containing heat-inactivated human serum.

The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications can be effected without departing from the true spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A monoclonal paratopic molecule that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118.

2. The paratopic molecule of claim 1 that is an intact antibody.

3. The paratopic molecule of claim 2 wherein said antibody is of the $IgG_3$ isotype.

4. A hybridoma having the ATCC accession number HB 9118.

5. A composition suitable for killing tumor cells having ganglioside GD2 expressed upon their cell surfaces that comprises a biologically active intact IgG3 monoclonal antibody that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118 as an active ingredient, said antibody being dispersed in an aqueous physiologically tolerable diluent in an amount sufficient to kill tumor cells that express ganglioside GD2 on their surfaces when said antibody and said tumor cells are admixed with either complement or effector cells.

6. The composition of claim 5 wherein said tumor cells are killed <u>in vitro</u>.

7. The composition of claim 5 wherein said tumor cells are of neuroectodermal origin.

8. The composition of claim 6 wherein said tumor cells are selected from the group consisting of neuroblastoma, glioma, melanoma and small cell lung carcinoma.

9. The composition of claim 5 wherein said monoclonal antibody is present in an amount sufficient to provide about 1 to 200 micrograms of said antibody to an aqueous composition containing

about $0.5x10^4$ to about $10x10^4$ tumor cells per milliliter.

10. A method for killing tumor cells that express ganglioside GD2 upon their cell surfaces that comprises admixing and contacting said tumor cells with (a) an aqueous composition containing a cytotoxic amount of biologically active intact IgG3 monoclonal antibody that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118 as an active ingredient; and (b) a cytotoxic amount of either or both of complement and effector cells.

11. The method of claim 10 wherein said contacting is carried out in vitro and effector cells are provided exogenously.

12. The method of claim 11 wherein said admixing and contacting are carried out at a concentration of said monoclonal antibody of about 1 to about 200 micrograms per milliliter with about $0.5x10^4$ to about $10x10^4$ tumor cells per milliliter.

13. The method of claim 10 wherein said tumor cells are of neuroectodermal origin.

14. The method of claim 13 wherein said tumor cells are selected from the group consisting of neuroblastoma, glioma, melanoma and small cell lung carcinoma.

15. The method of claim 10 wherein said contacting is carried out in vitro and complement is provided exogenously.

16. The method of claim 15 wherein said contacting and admixing are carried out at a concentration of said monoclonal antibody of about 1 to about 200 micrograms per milliliter with about $0.5x10^4$ to about $10x10^4$ tumor cells per milliliter.

17. A diagnostic system for assaying for the presence of ganglioside GD2, said system including in at least one container as an active ingredient, (1) an effective amount of a monoclonal paratopic molecule that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118, and (2) an indicating means for signalling the immunoreaction of the paratopic molecule with ganglioside GD2.

18. The diagnostic system of claim 17 wherein said indicating means is bonded to said paratopic molecule.

19. The diagnostic system of claim 17 wherein said paratopic molecule is an intact antibody.

20. A method for assaying for the presence of ganglioside GD2 in a sample comprising the steps of:

(a) admixing and contacting a sample to be assayed with an effective amount of a monoclonal paratopic molecule that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118;

(b) maintaining said contact under biological assay conditions for a predetermined time period for said paratopic molecule to immunologically bind to ganglioside GD2 present in the sample to form an immunocomplex; and

(c) determining the presence of said paratopic molecule that immunoreacted with ganglioside GD2.

21. The method of claim 20 wherein said assay is carried out <u>in vitro</u>.

22. The method of claim 21 wherein said assay is carried out in the solid phase and said

sample is affixed to a solid phase matrix prior to said admixing and contacting step.

23. The method of claim 22 wherein said monoclonal paratopic molecule is an intact antibody.

24. The method of claim 22 wherein said sample to be assayed is a tissue section.

25. The method of claim 22 wherein said sample to be assayed is a suspension of cells.

26. A solid phase assay method for detecting the presence of ganglioside GD2 in a sample comprising the steps of:

(a) providing a solid matrix on which to assay a sample;

(b) affixing a sample to be assayed to said solid matrix to form a solid phase support;

(c) admixing and contacting said solid phase support with a predetermined amount of an aqueous, liquid composition containing an effective amount of a monoclonal paratopic molecule that immunologically binds to ganglioside GD2 and is secreted by the hybridoma having the ATCC accession number HB 9118 to form a solid-liquid phase admixture;

(d) maintaining said solid-liquid phase admixture under biological assay conditions for a predetermined time sufficient for said paratopic molecule to immunoreact with ganglioside GD2 present on said solid support to form a solid phase immunoreactant;

(e) separating the solid and liquid phases; and

(f) determining the presence of immunoreactant in the separated solid phase so formed.

27. The method of claim 26 wherein said paratopic molecule is an intact antibody.

28. The method of claim 26 wherein said sample is a cell suspension.

29. The method of claim 26 wherein the presence of immunoreactant in the solid phase is determined by:

(i) admixing an effective amount of a biologically active second antibody with the solid phase immunoreactant to form a second solid-liquid admixture, said second antibody being capable of immunoreacting with said paratopic molecule while said paratopic molecule is bound in said solid phase;

(ii) maintaining the second solid-liquid admixture under biological assay conditions for a predetermined time period sufficient for said second antibody to immunologically bind to any of said paratopic molecule present in said solid phase immunoreactant to form a second solid phase immunoreactant;

(iii) separating the liquid from second solid phase immunoreactant so formed; and

(iv) assaying for the presence of any second solid phase immunoreactant so formed.

30. The method of claim 29 wherein said second antibody has an indicating means linked thereto.

31. The method of claim 30 wherein said indicating means is an enzyme.

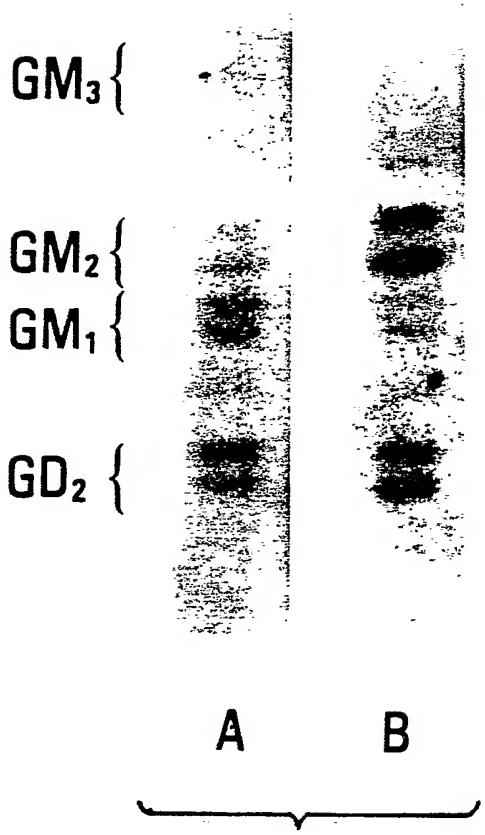


FIG. 1


FIG. 2

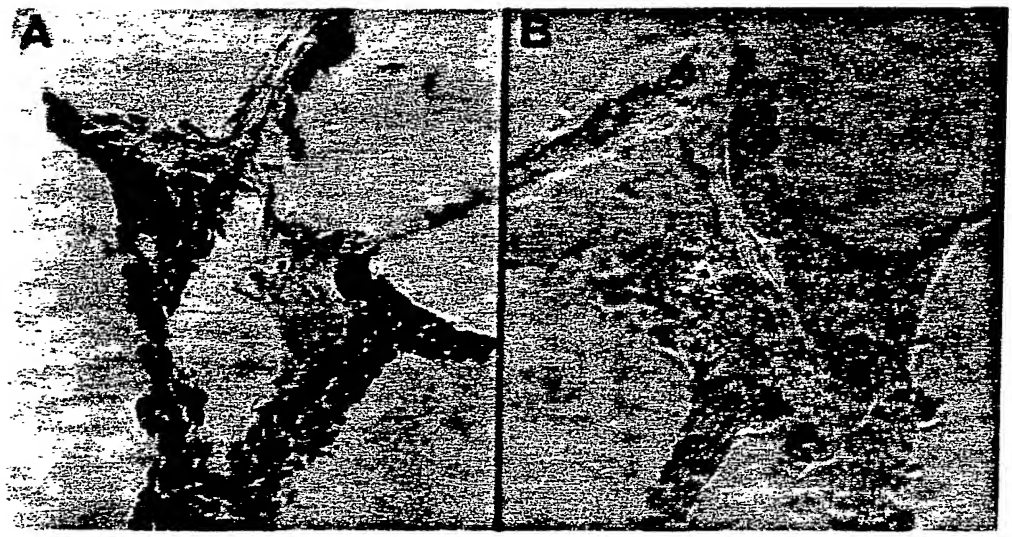


FIG. 3

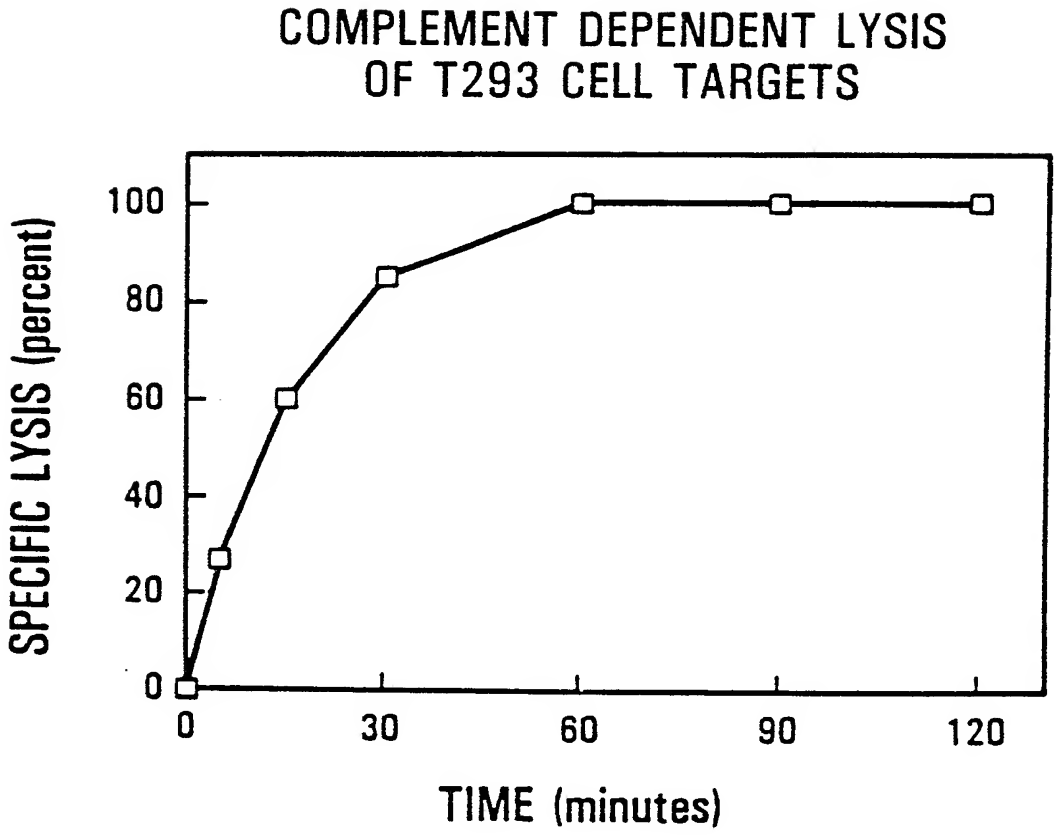
COMPLEMENT DEPENDENT LYSIS
OF T293 CELL TARGETS
SPECIFIC LYSIS (percent)
100
80
60
40
20
0
0
30
60
90
120
TIME (minutes)

FIG. 4

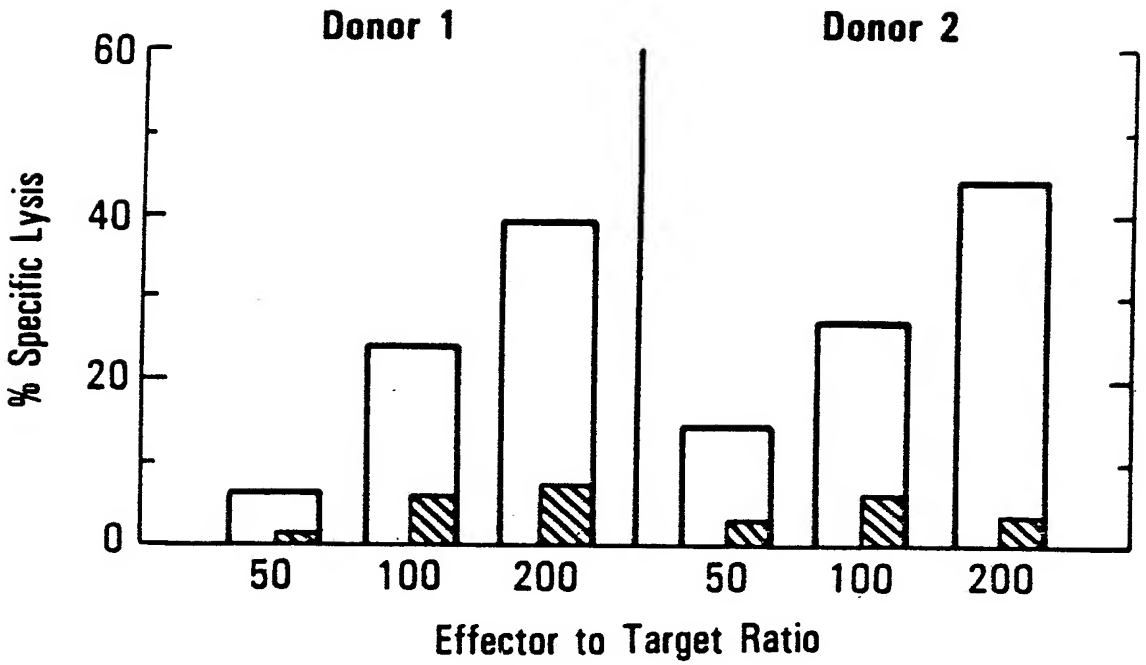
Donor 1
Donor 2
60
40
20
0
% Specific Lysis
50
100
200
50
100
200
Effector to Target Ratio

FIG. 5

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/02419

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/53,531,535,544,574,577; C12P 21/00

US CL :435/7;530/387,808,809;424/85;436/519,528,548,813;935/89, 95,106

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| US | 435/7; 530/387,808,809; 424/85; 436/519,528,548,813 935/89,95,106 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| Y,P | US, A, 4,675,287 (REISFELD) 23 June 1987, see column 3, line 20 to column 4, line 33; column 5, lines 6-23 and 27-34; column 16, lines 12-21; column 22, lines 4-18. | 1-31 |
| X<br>Y | Cancer Research, Volume 45, issued June 1985 (Baltimore), N.K. CHEUNG ET AL., "Monoclonal Antibodies to a Glycolipid Antigen on Human Neuroblastoma Cells", pages 2642-2649, entire document. | 1-10,13-16<br>17-31<br>11-12 |
| X | Biochemical and Biophysics Research Communications, Volume 127, issued February 1985 (New York), M. SAITO ET AL., "Ganglioside GD2 Specificity of Monoclonal Antibodies to Human Neuroblastoma Cell", pages 1-7, see abstract and page 2. | 1-4 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 16 November 1987 | 09 DEC 1987 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | Jack Spiegel |

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No [18] |
|---|---|---|
| Y | Proc. Natl. Acad. Sci. USA, Volume 79, issued September 1982 (Washington, D.C.), R. IRIE ET AL., "Human antibody to OFA-1, a Tumor Antigen, Produced in vitro by Epstein-Barr Virus-Transformed human B-lymphoid Cell Lines", pages 5666-5670. See pages 5669-5670. | 1-8,10 and 13-15 |
| Y | Proc. Natl. Acad. Sci. USA, Volume 79, issued December 1982 (Washington, D.C.), L. CAHAN, ET AL., "Identification of a Human Neuroectodermal Tumor Antigen (OFA-I-2) as Ganglioside GD2", pages 7629-7633, see page 7629 abstract and Materials and Methods. | 1-4 |
| Y | Cancer Research, Volume 44, issued December 1984 (Baltimore), G. SCHULZ, ET AL., "Detection of Ganglioside GD2 in Tumor Tissues and Sera of Neuroblastoma Patients" pages 5914-5920, see abstract and page 5919-Discussion. | 1-4 and 17-31 |
| Y | Proc. Natl. Acad. Sci USA, Volume 81, issued September 1984 (Washington, D.C) D. CHERESH, ET AL., "Localization of the Ganglioside GD2 and GD3 in Adhesion Plaques and on the Surface of Human Melanoma Cells", pages 5767-5771, see page 5769. | 1-4 and 17-31 |

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-16 drawn to compositions and method of body treating (killing cells); Class 424/89

II. Claims 17-31 drawn to compositions and methods of assaying/diagnosing; Class 436/548. See Attachment

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210, Part VI.1

Telephone Approval:

$140 payment approved by Edward P. Gamson on 30 October 1987 for Group II; Charge to Deposit Account No. 04-1644.

Counsel advised that he has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of mailing of the search report (Form 210).

Reasons for holding lack of unity of invention:

The invention as defined in Group I (claims 1-16), drawn to composition and methods of killing cells using same which is classified in Class 424 subclass 89 is a materially different and distinct process than the method of assaying and diagnosing of Group II (claims 17-31) classified in class 436 subclass 548.

Time Limit for Filing a Protest:

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack of unity only with respect to the group(s) paid for.